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THE BIOASSAY OF INSULIN BY THE COMPENSATION METHOD[1]

A. BRUCE MACALLUM

Abstract

The compensation principle developed by Bouckaert and de Duve (1) during an investigation of the action of insulin was modified for use as a method for the bioassay of insulin. This method has the advantage that the blood sugar level is maintained within physiological limits, thus eliminating the possibility that the action of the insulin might be modified by the elaboration in the test animal of homeostatic factors that regulate the blood sugar concentration.

Dry, crystalline zinc insulin preparations from three British and three American sources were investigated. Using the new method, the strength of each preparation in units per milligram was found to correspond with the strength of the international standard and also with the certified strength of the preparations as supplied by the manufacturers from results of assay by the pharmacopoeial method.

Introduction

During an investigation of the action of insulin, the compensation principle was developed by Bouckaert and de Duve (1). In this method the insulinized rabbit is perfused with a glucose–saline solution at a rate sufficient to compensate for the hypoglycaemia that would otherwise ensue. During the experimental period the blood sugar levels are maintained within physiological limits. Theoretically, it should be possible to replace the amount of glucose removed within a narrow range and in this manner to ascertain a value for its utilization. In practice, exact compensation is not always possible and the utilization values are obtained by correcting the amount of sugar infused by a factor dependent upon the observed values of blood glucose during the experimental period (1). A comprehensive review of the work of the above authors has already appeared (2).

The bioassay of insulin by the conventional hypoglycaemic method requires a large number of animals and, in the case of the rabbit method, numerous blood sugar determinations are necessary. If the compensation principle could be modified for use in the standardization of insulin preparations, a

[1]Manuscript received March 4, 1957.
Contribution from the Department of Biochemistry, University of Western Ontario, London, Ontario.

material reduction in the animal and analytical requirements would be achieved. Furthermore, in the compensation method, the blood sugar concentration is kept within physiological limits. This is of advantage, particularly when the action of substances antagonistic or synergistic to insulin is to be investigated. The following study was undertaken with the above objective in view.

Methods

During the course of the investigation, 545 experiments were carried out on 123 female rabbits between 2.5 and 5 kg. in weight. These were kept in the animal house for a month prior to use. Those infected with coccidiosis were rejected, since the livers of such animals were found to contain 10–40% of the normal content of glycogen. Each animal was kept in a separate cage and supplied with a diet of Master's Rabbit Meal (Toronto Elevators, Ltd.), supplemented with a weekly carrot ration. The animals were allowed unlimited access to water. It was felt that good housing and feeding are important factors in the securing of uniform results.

Bouckaert and de Duve (1) infused the glucose–saline by means of a gravity drip, the cannula being inserted in the marginal vein of the ear. The infusate was 4 or 6% glucose in normal saline. In order to obtain blood sugar values close to that of the initial sample, the flow was controlled using a flowmeter. In the present experiments the technique was simplified by fixing the volume of the infusate at 10 ml. per kg. of body weight per 80 minutes and adjusting the concentration of glucose so that the amount of glucose infused approximated the glucose utilization value anticipated under the conditions of the experiment. The gravity drip was replaced by a 50 ml. glass syringe, the plunger of which was driven by a small synchronous motor with a reducing gear and variable-speed control. In this way the amount of infusate could be regulated accurately in the range between 20 and 50 ml. per 80 minutes. The equipment is shown in Fig. 1. The cannula is a 20 gauge Huber point hypodermic needle inserted into the marginal ear vein and held in place by two 45 mm. artery clips.

The animal is shown with the neck loosely confined by a metal yoke extending over a notch in the box. Both yoke and notch are lined with ½ in. sponge rubber. Free movement of the head is desirable. The yoke should be just large enough to prevent withdrawal of the head. The legs and body must be free, since restraint stimulates a struggle and provokes a spontaneous hyperglycaemia.

Food is withheld from the animals 12 hours before the experiment.

At the beginning of the experiment, a blood sample is taken, after which the insulin is given intravenously and the infusion begun. A blood sample (0.1 ml.) is taken from the opposite ear at intervals of 20 minutes during the infusion period of 80 minutes. Glucose was determined by the method of Nelson (3).

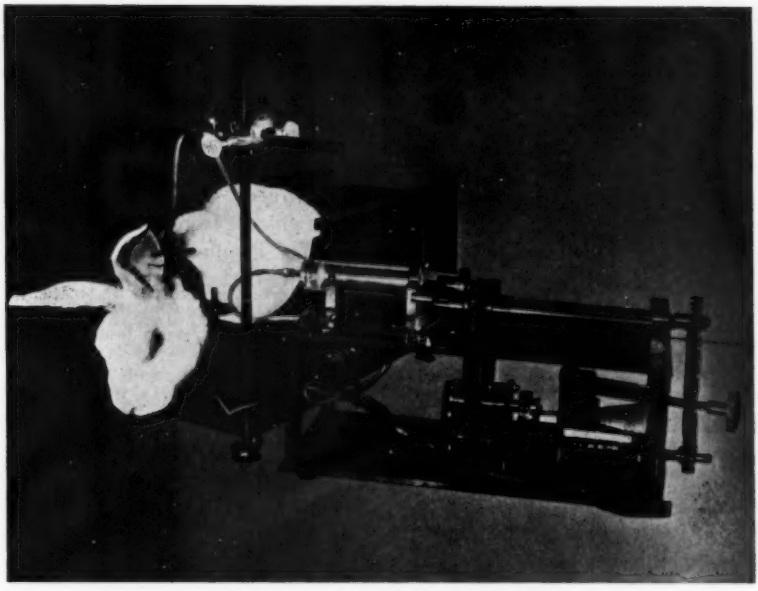

FIG. 1. Experimental arrangement.

Bouckaert and de Duve (1) used the following formula for calculating the glucose utilization value from the observed blood sugar figures:

$$D_0 = (D/G_1) \times G_0,$$

where D_0 = glucose utilization in g. per kg. per 80 minutes,
D = g. glucose infused per kg.,
G_1 = average of all blood sugar determinations,
G_0 = 125 mg. % blood sugar.

G_0 is an arbitrary figure, being the mean initial blood sugar value of a number of rabbits used in their laboratory. In the pharmacopoeial bioassay of insulin by the hypoglycaemic method, the initial blood sugar value for each animal is used in calculating the results. In the work reported here the initial blood sugar concentration was used instead of the figure given by Bouckaert and de Duve for G_0.

The above method of calculation of the glucose removed from the blood under the influence of insulin gives the most reproducible results when the blood sugar levels during infusion do not fall below 50 mg. % or rise above 160 mg. %. With few exceptions, all of the animals had initial blood sugar levels ranging between 70–110 mg. %. Within these limits, the calculated utilization value approximated that obtained when the infusion was repeated with the glucose concentration of the infusate adjusted so that the amount of glucose infused during the 80 minute period corresponded to the original

utilization value. Unless it is planned to study the behavior in any given animal, the blood samples taken during the infusion may be pooled and a single sample taken for duplicate analysis, thus reducing the time and labor involved.

Results

Bouckaert and de Duve (2) were interested in obtaining maximal utilization values. Consequently, the animal was 'saturated' by giving more than enough insulin to promote maximal removal of glucose from the blood. These workers reported that the 'saturation' dose of insulin was 6.25 units per animal. Although larger amounts were also used, the maximum utilization values were unchanged and remained at 1.07 ± 0.11 g. glucose per kg. per 80 minutes. They reported that the utilization values were less for smaller amounts of insulin.

As the writer was interested in the effect of factors synergistic or antagonistic to the action of insulin, a study was made of the rising portion of the dose–response curve. Table I shows the utilization values determined on a group of nine animals at four levels of insulin dosage ranging from 0.1 unit to 1 unit per kg. Subsequently, on this group and eight other animals, random checks (24 in all) at various insulin doses gave values falling within the confidence limits shown in the table.

TABLE I

RELATION OF GLUCOSE UTILIZATION TO INSULIN DOSAGE (G. PER KG. PER 80 MINUTES)

Insulin administered at start, followed by glucose infusion. Group of nine animals

Units of insulin per kg.	Glucose utilization (mean ± standard deviation)
0.1	0.212 ± 0.037
0.25	0.470 ± 0.069
0.50	0.821 ± 0.203
1.0	1.002 ± 0.176

The maximum, or 'saturated', glucose utilization value was not significantly different from that obtained when 1 unit of insulin per kg. was used, i.e. 1.002 ± 0.176 g. per kg. per 80 minutes. This figure agrees with that reported by Bouckaert and de Duve (1.07 ± 0.11).

These findings suggested that a detailed study of the utilization values for smaller amounts of insulin (0.1–0.25 units per kg.) in the rising portion of the dose–response curve would be more likely to be of use as a standardization procedure. It was also found desirable to work with dry, crystalline zinc insulin preparations, since these maintain their potency. Samples were secured from three British and three American sources. These preparations had been standardized by the suppliers, using the pharmacopoeial method. A certificate of the strength in units per mg. was furnished with each sample. Fresh solutions were made up from the dry preparation as required.

A change in the technique was introduced at this stage. Previously the insulin solution was given intravenously prior to the infusion. It was now added to the glucose–saline solution and administered with the infusion. This mixed infusion was found to give uniform results for amounts of insulin up to 0.25 units per kg., but the method could not be used with greater doses of insulin, where it was found necessary to administer the insulin separately to obtain the greatest reproducibility. In all subsequent work the mixed insulin–glucose infusion was used at an insulin dosage of 0.25 units per kg., or less.

In the experiments reported in Table II a group of 48 animals was used. This included three animals from the previous group of nine. Animals were selected at random from the whole group and two or three animals used for one insulin preparation were also used for others. Table II indicates that each of the mean glucose utilization values for the different products lies within the confidence limits of the mean value of the international standard. Small differences in the stated unit value per milligram were not reflected in the results. There was the possibility that the preservation of physiological blood sugar levels might reveal differences between preparations that were not apparent with the pharmacopoeial method, but no such difference appeared. Random checks with intermediate insulin dosages over a two-year period fell within the expected confidence limits.

TABLE II

GLUCOSE UTILIZATION VALUES (G. PER KG. PER 80 MINUTES)

Insulin infused with glucose

		0.1 units insulin per kg.		0.25 units insulin per kg.	
Insulin preparation	Units per mg. insulin preparation	No. of animals	Glucose utilization (Mean ± standard deviation)	No. of animals	Glucose utilization (Mean ± standard deviation)
A	International standard	17	0.206 ± 0.030	19	0.512 ± 0.048
B	23.2	8	0.213 ± 0.036	8	0.495 ± 0.052
C	23.6	8	0.207 ± 0.035	8	0.503 ± 0.054
D	22.7	8	0.207 ± 0.028	8	0.511 ± 0.046
E	27.0	8	0.200 ± 0.029	7	0.484 ± 0.041
F	23.0	8	0.205 ± 0.030	8	0.495 ± 0.045
G	23.3	8	0.202 ± 0.024	8	0.496 ± 0.042

Discussion

At high dosages, the insulin must be given before the infusion to get the maximum effect, and there is a wider variation from the mean. Some animals have a maximum capacity to utilize glucose in the range 0.6–0.7 g. glucose per kg. per 80 minutes and are useful only when the glucose utilization values are kept within the limits 0.2 to 0.5 g. per kg. per 80 minutes.

The low glucose utilization values (e.g. those given with 0.1 units per kg.) are remarkably constant during the useful life of the animal. Table III demonstrates the degree of reproducibility to be expected for individual animals over an extended time period. The table shows that the means and standard deviations remain similar to those reported in Table II.

A comparison of the standard deviations presented in Tables I and II indicates that, with an insulin dosage of less than 0.25 units per kg., the glucose–insulin mixtures give the more reproducible results. Under such conditions, where the sugar replacement is at or near the amount removed under the influence of the added insulin, it would appear that the elaboration of homeostatic factors regulating the blood sugar level is not stimulated.

TABLE III

GLUCOSE UTILIZATION VALUES (G. PER KG. PER 80 MINUTES)

Behavior of individual rabbits.* Insulin (0.1 units per kg.) infused with glucose

Rabbit No.	Experimental period (days)	Number of experiments	Glucose utilization (mean ± standard deviation)
114	440	5	0.239 ± 0.042
117	492	6	0.193 ± 0.019
120	399	5	0.190 ± 0.027
121	351	5	0.201 ± 0.047
123	121	5	0.211 ± 0.047
124	121	5	0.228 ± 0.033

*Six other rabbits showed a similar degree of variation.

Acknowledgments

The author is indebted to Messrs. Burroughs and Welcome, Boots, British Drug Houses, Sharpe and Dohme, E. R. Squibb, and Eli Lilly for supplies of crystalline zinc insulin. The international standard was furnished by the Connaught Laboratories, Toronto.

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REANIMATION OF RATS AFTER CARDIAC AND RESPIRATORY ARREST DUE TO LOW BODY TEMPERATURE[1]

JOHN HUNTER

Abstract

In confirmation of the report by Andjus (1951) it has been found possible to reanimate rats that have been cooled to 2°–3° C. and maintained at this temperature for periods up to 1 hour without cardiac or respiratory activity. The accumulation of metabolic carbon dioxide within the sealed vessel is an important part of the procedure if subsequent reanimation is to be attained. When the carbon dioxide is absorbed during the cooling phase, the animals remain active, their fall in body temperature is delayed and they do not recover from the hypothermia.

Preliminary rewarming of the chest was unnecessary in the reanimation procedures described but rapid rewarming of the animal seems advantageous. The animals do not recover unless they are ventilated artificially when they are being rewarmed.

Introduction

The respiratory and circulatory changes which occur with a fall in body temperature have been studied intensively for more than twenty years (8, 9, 10, 14, 15, 16, 17, 18, 20, 21). Until recently most of the evidence supported the conclusion that the cooling of mammals below 15° C. was fatal (1). That the heart beat and circulation could be re-established spontaneously in the rat after the body temperature had fallen to lower levels (2° or 3° C.), by heating the chest wall, was reported by Andjus in 1951 (2). He subsequently continued this work and modified the several phases in the cooling and reanimation procedure (3, 4, 5). Work in these laboratories was undertaken to verify the work reported by Andjus and to simplify, if possible, the reanimation procedures. A study has been made of some of the physiological changes occurring during the induction of low body temperature by this method. An attempt has been made to establish basic conditions and to clarify some of the physiological mechanisms.

Methods

Sixty female albino rats weighing between 200 and 300 g. were used in these studies. The method of induction of the hypothermia and the reanimation procedures are outlined separately under the description of the four methods used.

Method 1

Twenty female rats, between 200 and 240 g. body weight, were cooled in this series. Individual rats were sealed in a 2 liter jar containing room air. The exhaled carbon dioxide was allowed to accumulate. Each jar was surrounded by ice and placed in a domestic refrigerator (3° C.) for 1½ hours. At intervals during this period gas samples were analyzed for oxygen and

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Contribution from the Physiology Section, Defence Research Medical Laboratories, Box 62, Postal Station "K", Toronto, Ontario. This paper is Defence Research Medical Laboratories Report No. 109-1 on Project No. D50-93-10-72.

carbon dioxide content using a Fry analyzer (11). At the end of this period the rat was removed from the jar, the rectal temperature was recorded from an indwelling rectal thermometer, and the rat was wetted to expel air enmeshed in the fur. The rat was then placed in crushed ice with only its head and tail protruding. The rectal temperature was observed and recorded every 5 minutes. Cardiac activity was observed continuously by means of a cathode ray electrocardiograph (Smith and Stone, Model H-15). The rat was left in the crushed ice for 60 minutes after the last heart beat was observed on the electrocardiograph or 80–100 minutes after the rectal temperature fell below 15° C. The rat was then reanimated by rewarming in water at 45° C. and by artificial ventilation. The artificial ventilation was accomplished by enclosing the rat's head in a thistle tube, 2 in. in diameter, the mouth of which was covered with a taut rubber membrane provided with a hole for the rat's head (Fig. 1). The animals were artificially ventilated with 95% oxygen– 5% carbon dioxide. The oxygen – carbon dioxide mixture was delivered from a cylinder to the thistle tube. A side tube from the latter was periodically closed in order to increase the pressure within and ventilate the rat. The pressure changes were from atmospheric to +10 mm.Hg. This upper limit was regulated by placing the open end of another side tube 10 mm. below the surface of a beaker of mercury. Expiration was aided by compressing the chest manually. Reanimation efforts were continued until the rat breathed spontaneously and regularly. The animal was then transferred to a small lucite chamber which was continuously flushed with the oxygen – carbon dioxide mixture. This chamber was immersed in a pan of warm water. When it had sufficiently recovered to assume an upright position, the rat was transferred to the animal colony.

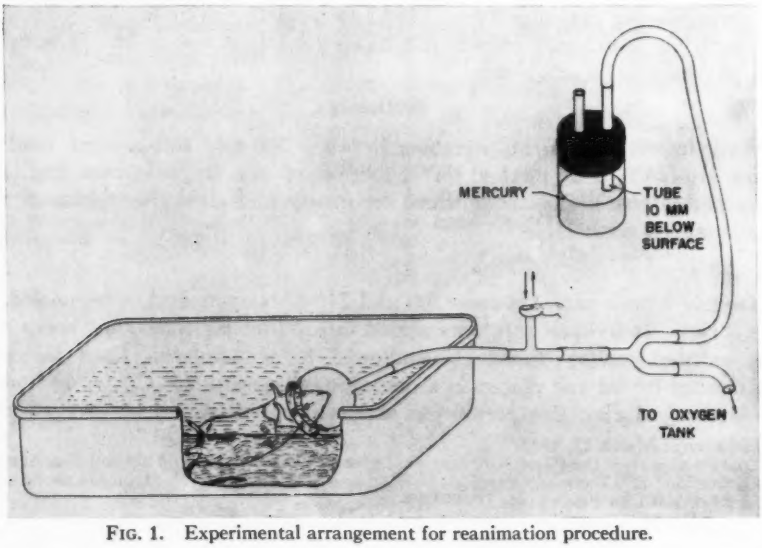

FIG. 1. Experimental arrangement for reanimation procedure.

Method 2

Ten female rats, between 200 and 320 g. body weight, were treated as in Method 1 but they were maintained in the crushed ice for a shorter period, namely 10–40 minutes after cardiac arrest or 25–60 minutes after rectal temperatures fell below 15° C. The rats were rewarmed slowly in room air. The procedure for artificial ventilation was similar to that in Method 1 except that air was used instead of the oxygen – carbon dioxide mixture.

Method 3

Ten female rats, between 195 and 240 g. body weight, were treated as in Method 1 with the following changes: The jars were initially flushed with 95% oxygen rather than with air. During the period in the jar the carbon dioxide initially present and the expired carbon dioxide were largely absorbed by soda lime and did not accumulate as in the preceding methods. This was the only procedure in which there was a major change in the method of induction of the hypothermia.

Method 4

Ten female rats, 250 to 310 g. body weight, were treated as in Method 1 and 2 but, during rewarming, artificial ventilation was omitted.

Results

Reanimation by Artificial Ventilation and Rapid Rewarming of Rats Exposed to High Carbon Dioxide Concentrations during Cooling (Method 1)

The results in this series are shown in Table I. The solid line in Fig. 2 indicates the changes in rectal temperature for one individual rat of this

TABLE I

METHOD 1

Body wt., g.	Initial rectal temp., ° C.	Rectal temp. after 1½ hr., ° C.	CO_2 after 1½ hr., %	Rectal temp. at last heart action	Total cardiac arrest, min.	Recovery	Remarks
240	35.0	20.0	7.3	7.0	65	Yes	
205	34.5	31.0	—	6.0	70	Yes	
220	37.0	21.0	9.0	4.5	62	Yes	
205	33.5	21.5	11.1	8.5	70	Yes	Rat died 1 week later
210	33.5	21.0	11.8	7.5	70	Yes	
210	35.5	20.0	10.9	5.5	75	Yes	
220	36.0	22.0	11.2	5.0	70	Yes	
210	34.5	20.5	3.8	4.0	70	Yes	
235	34.5	19.5	10.7	5.5	—	No	
235	35.5	19.0	14.0	6.0	70	Yes	
215	36.0	23.5	12.1	8.0	—	No	
230	35.0	21.5	10.1	6.5	—	No	
235	36.5	24.0	8.4	6.0	—	No	
230	35.0	22.5	10.3	5.5	70	Yes	
220	35.0	20.0	13.0	5.0	70	Yes	
215	36.5	24.0	8.2	7.0	70	Yes	
200	35.0	19.0	9.4	4.0	10	Yes	
200	31.5	22.0	12.7	5.0	30	Yes	
240	34.5	21.0	15.0	6.5	—	No	
225	35.5	21.0	11.0	5.0	40	Yes	
Av. 220	35.0	21.5	10.5	5.9			

group. Differences in the effects of slow and rapid rewarming are evident in this chart. The rectal temperature fell from an initial average value of 35° C. to 21° C. during the cooling phase in the sealed jar. Meanwhile carbon dioxide gradually accumulated to reach a final average concentration of 10.5%. The rats moved around actively for the first 30–35 minutes and then remained quietly hunched up. After the rats had been 30 minutes in the jar a number of air analyses indicated utilization of one-half the total oxygen and the accumulation of an equivalent volume of carbon dioxide. From 30 minutes onwards additional oxygen utilization was much less. The rats offered only mild resistance to their introduction to the crushed ice. When they were surrounded by ice, their rectal temperatures fell precipitously. At a rectal temperature of 15° C. regular breathing had stopped but occasional gasps separated by lengthy intervals were observed down to a 10° C. rectal temperature. Below 10° C. cardiac rhythm was lost, but isolated beats, separated by intervals of ½–2 minutes were observed in a few rats at rectal temperatures of 5°–6° C. At the time of the last heart beat the average rectal temperature was 5.9° C. With further cooling the rectal temperature fell to between 0° to 2° C.

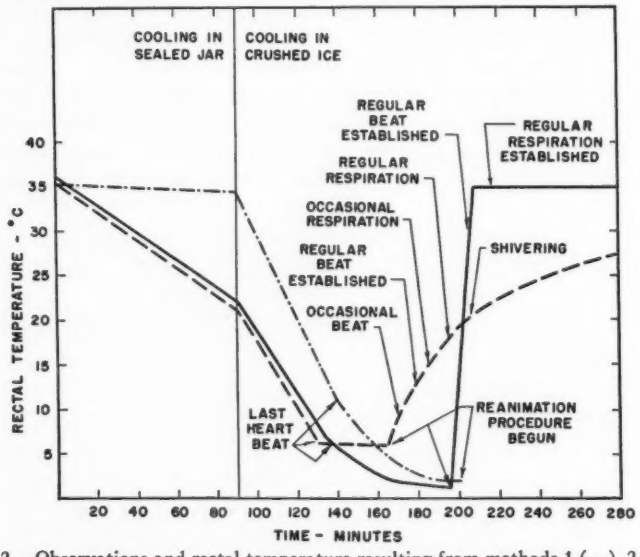

FIG. 2. Observations and rectal temperature resulting from methods 1 (—), 2 (— —), and 3 (• —).

After the application of heat combined with artificial ventilation, rhythmical cardiac activity began spontaneously after 6–10 minutes. Regular breathing was evident 20–30 minutes after the start of the rewarming procedure. Further spontaneous recovery from the limp and comatose state was achieved in the lucite chamber flushed with 95% oxygen – 5% carbon dioxide, where the rats assumed a normal posture within 30–60 minutes.

Of the rats treated in this way, 75% recovered without apparent ill effects and were alive 7 months after treatment.

Reanimation, by Artificial Ventilation and Slow Rewarming in Room Air, of Rats Exposed to High Carbon Dioxide Concentrations during Induction of Hypothermia (Method 2)

The results obtained in this series of 10 rats are shown in Table II, and the typical differences between slow and rapid rewarming are illustrated in Fig. 2.

TABLE II

METHOD 2

Body wt., g.	Initial rectal temp., ° C.	Rectal temp. after 1½ hr., ° C.	Time in crushed ice below 15° C. until start of rewarming, min.	Total cardiac arrest, min.	Recovery	Remarks
275*	36.5	22.0	30	15	Yes	Animal reanimated—regular heart action and regular breathing. Died later. Autopsy—lung damage, hemorrhagic bowel
275*	36.5	25.0	20	20	Yes	Apparent healthy recovery except weakness in right legs. Laparotomy—no evidence of hemorrhage or necrosis. Subsequent complete recovery
320*	37.0	26.5	30	15	Yes	
210	34.5	21.5	30	15	Yes	
250	35.0	21.0	30	20	No	Occasional heart beat observed but animal did not recover
200	35.5	24.5	45	35	Yes	Uneventful recovery
220	35.0	19.0	60	60	Yes	Animal started to make an excellent recovery (good heart beat and resp.) but subsequently died. Autopsy—hemorrhagic patches in lungs
200	35.0	20.0	60	25	Yes	
210	35.5	19.0	60	50	No	An occasional heartbeat was observed but animal did not recover
220	35.5	20.0	60	45	Yes	Uneventful recovery

*Rats used in previous reanimation procedures.

This group was kept chilled for a shorter period than in the previous series. Artificial ventilation was maintained for a period of 40 minutes as compared with the 10 minute period required for the rapidly rewarmed group. Rectal temperatures rose more slowly. A regular heart beat was usually established at a rectal temperature of 13° C.–15° C. in this group. The first spontaneous inspiratory effort appeared at a lower rectal temperature than in rats rapidly rewarmed but a regular rapid rhythm was not established until the rectal temperature was 15° C.–20° C. The experimental procedure used had the effect of shortening the period of cardiac arrest as rewarming was begun sooner than in Method 1. Eight of the 10 rats treated were reanimated but two died several hours later.

Attempted Reanimation of Rats by Artificial Ventilation and Rapid Rewarming. No Accumulation of Carbon Dioxide Allowed during Cooling (Method 3)

The results obtained in this series of 10 rats are shown in Table III. The most striking differences between this and the preceding series were: (*a*) Little

TABLE III

METHOD 3

Body wt., g.	Initial rectal temp. ° C.	Rectal temp. after 1½ hr., ° C.	CO_2 after 1½ hr., %	Rectal temp. at last heart action	Recovery
210	36.0	32.0	1.9	9.0	No
195	35.5	34.5	1.9	6.0	No
215	37.0	36.0	2.4	8.5	No
200	37.0	36.5	1.9	11.5	No
210	36.5	37.0	3.0	5.0	Yes Residual weakness in hind legs
240	35.5	36.0	2.0	7.5	No
225	36.5	34.0	1.9	7.5	No
215	35.0	34.5	1.7	11.0	No
215	35.0	35.5	1.9	9.0	No
205	35.5	34.5	1.8	9.0	No
Average 207	36.1	35.0	1.9	8.4	10% recovery

or no fall in rectal temperature took place during the 1½ hour period of exposure to cold in the sealed jar. The rectal remperatures of one rat in this series are plotted in Fig. 2. (*b*) The rats remained active throughout the period in the closed container. (*c*) Cardiac arrest occurred at a higher rectal temperature in the crushed ice. (*d*) Nine of the 10 rats so treated died.

Attempted Reanimation without Artificial Ventilation (Method 4)

In a final series of 10 rats, no effort was made to ventilate the animals artificially after removal from the crushed ice. Otherwise the treatment was the same as in Methods 1 and 2. No animals, whether warmed rapidly or slowly, survived without artificial ventilation (Table IV).

Acute Experiments on Blood Gas Content

The concentration of carbon dioxide in venous blood was determined in a number of rats exposed to carbon dioxide concentrations as in Method 1 (19). The average of the initial values was 39.8 volumes %. After 1½ hours in the sealed jar, the average value was 66.6 volumes %.

TABLE IV

METHOD 4

Body wt., g.	Initial rectal temp., ° C.	Rectal temp. after 1½ hr. ° C.	CO_2 after 1½ hr., %	Temp. at last regular cardiac rhythm*	Remarks
275	36.5	22.0	11.8	10.0	Rats placed in warm water at 40° C. Spontaneous ventricular beats were observed in all rats but none recovered
305	36.0	22.0	11.4	11.5	
290	37.0	21.5	13.7	14.0	
275	36.5	19.0	11.4	—	
255	35.0	20.0	11.5	11.0	
285	36.0	22.5	13.0	9.5	Rats slowly rewarmed in room air. No heart action was observed during this period. No rats recovered
300	35.0	22.0	14.0	10.0	
250	35.5	22.5	11.6	9.5	
290	36.0	22.0	14.0	8.5	
270	37.0	20.0	13.1	8.5	
Average values					
280	36.0	21.4	12.5	9.25	

*This temperature is discussed under Method 1 results.

Discussion

An initial rewarming of the chest to initiate the heart beat before rewarming of the remainder of the body was considered by Andjus and his colleagues to be imperative. In this way it was felt that the metabolism of the tissues would be kept as low as possible until a functioning heart could re-establish blood flow. Andjus accomplished this initial rewarming of the chest by (*a*) applying hot metal spatulas to the chest, (*b*) by focusing a beam of light on the chest, and (*c*) by heating the chest by microwave diathermy. While such action is based on sound principles, it seems that the danger of tissue damage by anoxia has been exaggerated and that general rapid body warming combined with artificial ventilation will reanimate rats.

In these experiments, a high carbon dioxide level in the respired air during cooling is shown to be essential to the ultimate success in reviving the animals. It is of interest here that in the absence of carbon dioxide accumulation, under the conditions of these experiments, the fall in rectal temperature during the induction phase before the animals were placed in the crushed ice was prevented.

Barbour and Seevers have reported that, during narcosis induced by carbon dioxide and cold, voluntary movement ceases before a body temperature of 23° C. is reached, indicating additional depression of the animal by carbon dioxide (6). Comparison of the activity of our animals with and without carbon dioxide accumulation during the period in the sealed jar is in perfect agreement with this. The work of Barbour and Seevers helps to explain the efficacy of the carbon dioxide induction in hypothermia. They point out that a sudden and well marked increase in the tension of carbon dioxide in the tissues produces a definite, if temporary, decrease in the total oxidative

metabolism and a parallel reduction in the activity of certain body tissues, including some portions of the nervous system. A decrease in total oxygen consumption in the presence of carbon dioxide (10–20%) has also been reported by these authors (7). Carbon dioxide excess, therefore, both accelerates cooling and diminishes oxygen consumption. The effects of hypothermia and of carbon dioxide on oxygen usage are therefore complementary. With a respiratory acidosis, a shift in the oxygen dissociation curve to the right takes place; oxygen release from hemoglobin is facilitated.

The circulatory effects of a high concentration of carbon dioxide have been well documented (13). These include depression of atrioventricular conduction or complete heart block, greater distensibility of the myocardium, increased venous return, and a marked increase in cerebral blood flow. Certain anoxic symptoms are preventible by carbon dioxide. For example, Gellhorn and Steek in studies of the effect on peripheral blood flow of gas mixtures containing low oxygen, with or without increased carbon dioxide tension, demonstrated that where the carbon dioxide tension was increased there was no compensatory increase in blood flow on readmission to air (12).

During rewarming with artificial ventilation, the effects of cooling and a high carbon dioxide level should be rapidly reversed. The body temperature rises and excess carbon dioxide is blown off. In our experience, a high oxygen concentration in the respired mixture during this period when cardiac and respiratory activities are subnormal produced better results than when air was given. Residual paralyses and delayed deaths were more common when the ventilation was carried out with air during the reanimation procedure.

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CHOLESTEROL BIOSYNTHESIS: THE STARVATION BLOCK[1]

J. F. Scaife and B. B. Migicovsky

Abstract

Partial localization of the metabolic block in cholesterol biosynthesis from acetate by starved rat liver homogenates has been achieved. Experimental evidence indicates that this block is located in the biosynthetic pathway between β-hydroxy-β-methyl glutaric acid and squalene. Fractionation and comparative chromatographic examination of incubated homogenates from starved and normal rats failed to reveal any accumulation of an appreciably radioactive intermediate as a result of the blocked biosynthetic pathway in the starved animal. A strongly labelled acidic compound has been isolated in minute amounts from incubated homogenates of both starved and normal rats. This is readily incorporated into cholesterol by liver homogenates from normal, but not from starved rats. Its identity has as yet not been established.

Introduction

The failure of liver homogenates from starved rats to incorporate acetate into cholesterol has been previously demonstrated (12). Acetate is incorporated into fatty acids by such homogenates, but to a lesser extent than with homogenates prepared from normal animals (21). It becomes pertinent to establish where, in the pathway leading from acetate to cholesterol, the starvation block exists. When rats are starved, incorporation of acetate into the di- and tri-carboxylic acids of the Kreb cycle by liver slices (9) is reduced and conversion of 1-C^{14}-acetate to $C^{14}O_2$ by liver homogenates is decreased (21). The possibility exists therefore that an accumulation of some intermediate might occur owing to the blocked cholesterogenesis in homogenates from starved animals.

Recent work has postulated the nature of several intermediates in acetate cholesterogenesis. A likely initial precursor is acetoacetate (5, 6), formed by condensation of acetate with acetyl-S-CoA. Further condensation would lead to a four carbon intermediate, necessary to form the repeating isoprenoid skeleton which is believed to be the building unit of most steroids. 2-C^{14}-Acetate has been found to be incorporated into β-hydroxy-β-methyl glutaric acid (HMG) and the labelled HMG has been shown to be incorporated into cholesterol (3, 16, 17, 19). Other proposed intermediates are β,β-dimethyl acrylic acid (DMA) (14, 16, 20), β-hydroxyisovaleric acid (15), and β-methyl glutaconic acid (16). The greater ease of incorporation of β-hydroxy-β-methyl-δ-valerolactone into cholesterol, as compared with HMG or DMA, has been cited as evidence that this compound is more nearly a direct intermediate of cholesterogenesis than the other two (22, 23).

A higher intermediate may be farnesenic acid, which has been isolated as the labelled derivative after acetate incubation (7).

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Because of its structure, squalene has been favored as a likely intermediate, and considerable evidence makes this probable. It has been isolated as a labelled compound by trapping procedures (11), and shown to be readily converted to cholesterol by liver preparations (10). Both *in vitro* and *in vivo* it reduces the incorporation of acetate to cholesterol (11).

By using techniques similar to the above, the highly specific dietary requirement for cholesterol of *Dermestes vulpinus* larvae has been shown to be due to a block in the normal cholesterogenesis mechanism (4) occurring after the formation of squalene from acetate.

A study has been undertaken to determine the location of the block in acetate cholesterogenesis produced in rat liver homogenates by starvation. In addition, a direct search by comparative chromatographic techniques, both partition and adsorption, has been made for the accumulation of a labelled intermediate caused by this block.

In this paper homogenates prepared from normal and starved rats will be referred to as "normal" and "starved" homogenates.

Materials and Methods

The technique for the preparation of liver homogenates, incubation, and measurement of acetate incorporation into cholesterol has already been described (12). All acetate used in this work was 1-C^{14}-sodium acetate.

Large scale incubations of pooled liver homogenates from 25 rats were achieved in a shallow Pyrex dish. A modified Dubnoff shaker was used and conditions were identical with those described above for the small scale incubations.

C^{14}-Acetoacetate was measured by the modified method (1) of Jowett and Quastel (13).

C^{14}-Squalene (7, 10) and C^{14}-HMG (17) were prepared and isolated as described previously. Squalene was the natural biosynthetic isomer, and was added to the homogenate mixture at levels of 5 mg./ml. as an emulsion in 2% gelatine solution. HMG was added at levels of 2 mg./ml. of homogenate. The isolated C^{14}-squalene was chromatographed on silicic acid plates using chloroform as solvent and the activity identified with the squalene. Chromatography of the C^{14}-HMG on paper (C_2H_5OH, 8: NH_3, 1: H_2O, 1) likewise identified the activity with the acid.

Silicic acid plate chromatograms were prepared as outlined by Rigby and Bethune (18).

All compounds added directly to the homogenate mixtures were adjusted to the pH of the homogenizing buffer (7, 2) beforehand.

Autoradiograms were prepared by direct contact with Kodak Industrial X-ray film, Type AA.

The extraction procedure for large scale incubates was as follows: The incubated homogenate mixture was killed by the addition of two volumes of 95% ethyl alcohol and one-fifth its volume weight of potassium hydroxide. After 2 hours on the steam bath, the excess alcohol was removed by a current

of nitrogen and the non-saponifiable fraction extracted with petroleum ether (b.p. 40°–60° C.). The mixture was then acidified with hydrochloric acid and extracted with the same solvent several times to remove fatty acids. Both petroleum ether extracts were washed thoroughly with distilled water. The acidified mixture was then extracted with chloroform twice, followed by repeated extraction with peroxide-free diethyl ether. The chloroform extracts were washed with water, filtered, and concentrated on the steam bath. The pooled ether extracts were dried overnight with anhydrous sodium sulphate before they were taken to dryness, the last of the solvent being removed under vacuum at room temperature.

Results

Localization of the Starvation Block

As an initial step, the ability of both "starved" and "normal" homogenates to form acetoacetate from 1-C^{14}-sodium acetate was examined. The results of this experiment are shown in Table I and it is apparent that the "starved" homogenate is able to incorporate acetate into acetoacetate as efficiently as the "normal" preparation. The labelled acetoacetate was located almost exclusively in the supernate (30 minutes at 140,000 × *g*) of the incubated homogenate. Addition of acetoacetate in relatively large amounts reduced the incorporation of acetate into cholesterol (Table II).

TABLE I

ACETOACETIC ACID PRODUCTION BY "STARVED" AND "NORMAL" LIVER HOMOGENATES*

(1.0 ml. homogenate; 2 hours' incubation)

	μM. acetate incorp. × 10^3	Total production, μM.
"Starved"	800	2.6
"Normal"	810	1.6

*Each value is the mean of three separate determinations.

TABLE II

EFFECT OF ACETOACETATE ON ACETATE INCORPORATION INTO CHOLESTEROL

(1.5 ml. liver homogenate; total volume 2.4 ml.)

Sodium acetoacetate, mg.	0	0.5	1	5	10
μM. acetate incorp. × 10^3	78.7	75.5	70.6	52.3	40.5

TABLE III

EFFECT OF HMG AND DMA ON ACETATE INCORPORATION INTO CHOLESTEROL

(1.5 ml. liver homogenate; total volume 2.4 ml.)

HMG, mg.	0	0.5	1	5	10
μM. acetate incorp. × 10^3	37.9	28.5	21.6	11.4	8.4
DMA, mg.	0	0.5	1	2.5	5
μM. acetate incorp. × 10^3	73	52	49	29	4

TABLE IV

INCORPORATION OF ACETATE INTO HMG BY "NORMAL" AND "STARVED" LIVER HOMOGENATES

(1 ml. homogenate: HMG added at 2 mg./ml. homogenate)

	Acetate incorp., μM. $\times 10^3$	Total HMG, mg.	
		Added	Recovered
"Normal"	105	80	60
"Starved"	82	72	50

TABLE V

EFFECT OF SQUALENE ON ACETATE INCORPORATION INTO CHOLESTEROL

(1.5 ml. "normal" liver homogenate; total volume 2.4 ml.)

Squalene, mg.	0	0.5	1	2	5	10
μM. acetate incorp. $\times 10^3$	47	42	38	31	20	12

TABLE VI

INCORPORATION OF ACETATE INTO SQUALENE

(1 ml. "normal" liver homogenate)

Squalene added, mg./ml.	2	5	8
μM. acetate incorp. $\times 10^3$	1.0	1.4	2.6

TABLE VII

INCORPORATION OF SQUALENE INTO CHOLESTEROL BY "NORMAL" AND "STARVED" LIVER HOMOGENATES

(1 ml. homogenate; squalene added, 2 mg./ml. homogenate)

	Squalene incorp. into cholesterol, μM. $\times 10^3$	Squalene added, c.p.m./μM.	Cholesterol recovered, c.p.m./μM.
"Normal"	52	347	35
"Starved"	0	347	0

TABLE VIII

INCORPORATION OF 1-C^{14}-ACETATE AND 1-C^{14}-DMA INTO CHOLESTEROL BY "NORMAL" AND "STARVED" LIVER HOMOGENATES

(1.5 ml. homogenate; total volume 2.4 ml.)

	Acetate added, 4 μM.	DMA added, 4 μM.
	μM. incorp. $\times 10^3$	
"Normal"	73	0.21
"Starved"	0	0

The addition of HMG to "normal" homogenates produced a marked reduction in the incorporation of acetate into cholesterol (Table III). Isolation of added HMG (2 mg./ml. of homogenate), from both "starved" and "normal" incubated homogenates revealed that the compound was appreciably labelled in both cases, as is demonstrated in Table IV.

The addition of natural squalene to homogenates markedly reduced the incorporation of acetate into cholesterol (Table V). Isolation of added squalene from "normal" incubated homogenates revealed the trapping of considerable amounts of labelled squalene (Table VI). With "starved" homogenates similar additions and subsequent isolation of squalene failed to reveal the presence of any labelled molecules.

Incubation of "normal" homogenates with labelled squalene, isolated by the above procedure, led to the formation of labelled cholesterol (Table VII). Under identical conditions using "starved" homogenates no cholesterol was produced from labelled squalene.

The effect produced by increasing quantities of DMA upon acetate incorporation into cholesterol by "normal" homogenates is given in Table III. A comparison of 1-C^{14}-acetate, and 1-C^{14}-DMA incorporation into cholesterol by "normal" and "starved" homogenates is given in Table VIII.

Isolation of Intermediates

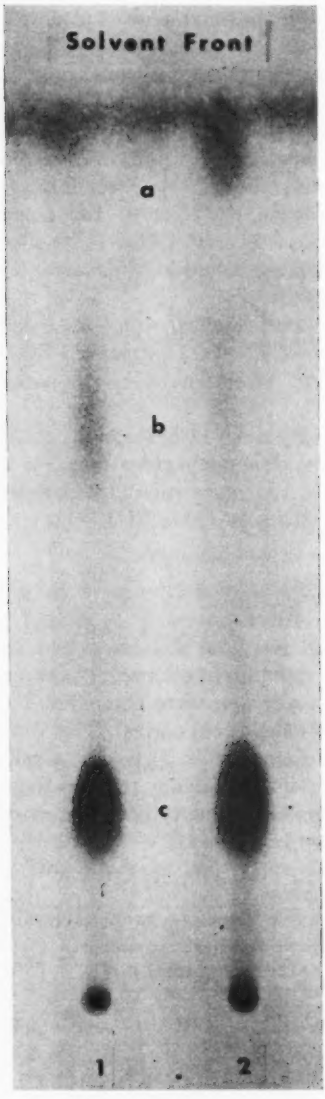
Large scale incubations of pooled rat liver homogenates were attempted in order to locate an accumulation of an active intermediate caused by the starvation block of cholesterogenesis from acetate. The incubated mixtures were treated as described above and separated into fractions. These fractions, from both "starved" and "normal" homogenates, were compared by paper partition and adsorption chromatography (silicic acid plates). No compound peculiar to the "starved" homogenates that was in any way significantly labelled was located in any of the fractions (including the residue). The distribution of C^{14} throughout these fractions for typical "starved" and "normal" incubated homogenates is shown in Table IX.

TABLE IX

DISTRIBUTION OF C^{14}-ACTIVITY IN SEPARATED LIVER INCUBATE FRACTIONS

	Per cent of recovered activity				
Fraction	Non-sap. fraction	Pet. ether ext. of acidified soln.	$CHCl_3$	Diethyl ether	Residue
"Normal"	8.5	1.6	30.6	42.2	17.0
"Starved"	0	8.7	32.6	41.3	17.4

Fig. 1 shows a comparison of the non-saponifiable fractions obtained from "starved" and "normal" homogenates chromatographed on silicic acid. Screening revealed that the C^{14} activity of the "normal" fraction was located almost completely in the cholesterol subfraction, while the total "starved" fraction was almost completely devoid of C^{14} activity.



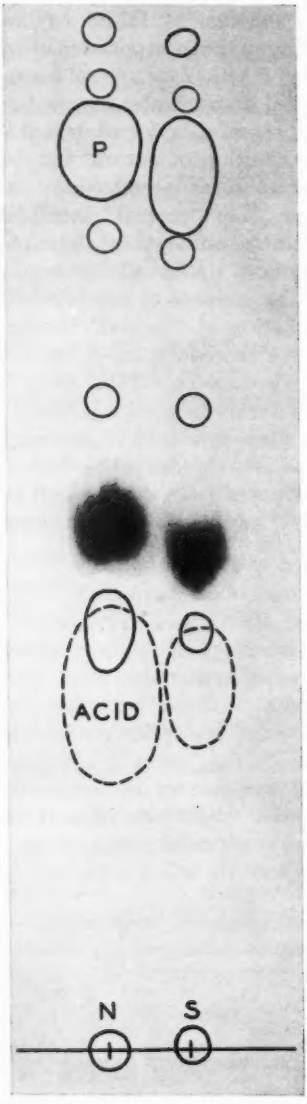


FIG. 1. Silicic acid chromatogram of non-saponifiable fractions from "starved" and "normal" liver homogenates; 1 = "normal", 2 = "starved", C = cholesterol.

FIG. 2. Diethyl ether fractions of incubated liver homogenates on a paper chromatogram (Whatman 3MM) and (*n*-butanol: 1.5 *N* NH_3) with the superimposed radioautogram showing the relative position of the C^{14}-containing compound *x*;
N = "normal", S = "starved", O = fluorescent material.
P = purple fluorescent material, O = acid resisting non-fluorescent material, darkened spots are the autograms of compound *x*.

Examination by paper chromatography (*n*-butanol: 1.5 *N* NH_3) of the chloroform and diethyl ether fractions from both "starved" and "normal" incubates revealed that the activity was almost completely located in one position on the chromatogram. This compound (or compounds) *x* is identical in both fractions. A preliminary chloroform extraction is, however, necessary to free the mixture of large amounts of intractable non-labelled gums, which make chromatographic separation difficult. Examination of the diethyl ether fraction by ultraviolet light (230–415 mμ) after the above chromatographic separation revealed the presence of six other compounds. The major component of these compounds fluoresced deep purple under 253 mμ radiation. It has been shown not to be ATP, DPN, or nicotinamide. None of these fluorescent materials showed any appreciable C^{14} activity. In addition, spraying with bromcresol green indicator revealed the presence of an appreciable quantity of a weakly labelled acid. The C^{14}-active material *x* also showed an acidic reaction. These findings are demonstrated in Fig. 2.

Elution of *x* from the paper by acetone produced a small amount of material (approximately 1 mg. from an incubation of 25 livers). Paper chromatographic examination (*n*-butanol: 1.5 *N* NH_3) of this material with the acids of the tricarboxylic acid cycle (citric–oxalacetic) and with HMG, DMA, β-hydroxy butyric, α-keto butyric showed it was none of these.

The material *x* was volatile at 100° C. and was kept under nitrogen at −20° C. On paper electrophoresis (8) it behaved as an anion. Under identical conditions the accompanying purple fluorescent material mentioned above was positively charged. Material *x* gave a major peak in the ultraviolet spectrum at 274 mμ and a subsidiary one at 280 mμ. Infrared examination revealed the presence of a strong C=O bond compatible with a —COOH group, the probable presence of an independent non-hydrogen-bonded —OH group, and a *cis* C=C grouping.

Compound *x* can be readily incorporated into cholesterol in "normal" but not in "starved" homogenates (Table X). The addition of HMG to "normal" homogenates reduces the incorporation of *x* into cholesterol.

TABLE X

COMPARISON OF INCORPORATION INTO CHOLESTEROL OF COMPOUND *x* AND ACETATE
(1.5 ml. liver homogenate; total volume 2.4 ml.)

	Compound *x*		Acetate		
Sample*	c.p.m. added	% incorp.	Conc.	c.p.m. added	% incorp.
1N	87.4 × 10^4	0.5	1 μM.	15.6 × 10^6	1.7
1S	"	0			
2N	57 × 10^4	0.3	"	"	2.0
2S	"	0			
3N	72 × 10^4	0.9	"	"	2.1
3S	"	0			

(Starved incorp. = zero for each preparation.)
*N = "normal", S = "starved".

Discussion

It is difficult, using the above criteria of trapping, isolation, and comparative degree of incorporation of labelled substrates, to deduce infallible proof that any particular compound is a direct intermediate in cholesterogenesis from acetate. The reduced incorporation of a substrate, because of the addition of another material, cannot be interpreted unequivocally as a trapping effect produced by the addition of that material. An inhibition will achieve the same effect. This is illustrated by the results in Tables III and VIII. Although DMA reduces substantially acetate incorporation into cholesterol, it is not itself, at least not in total, incorporated into cholesterol by this system. Hence DMA cannot be the ultimate isoprene unit incorporated finally into the steroid nucleus.

Acetoacetate has been shown (5, 6) to be readily incorporated into cholesterol without prior breakdown to acetate and is formed in large quantities from acetate by liver slices (9). Formation of acetoacetate from other fatty acids has also been demonstrated (13). The present results show there is no hindrance to the formation of acetoacetate from acetate in "starved" homogenates as compared with "normal" homogenates.

Squalene has been considered by several workers (10, 11) to be a likely direct intermediate in cholesterogenesis from acetate and even deducing from its structure alone makes this seem probable. Although C^{14}-squalene has been isolated from "normal" homogenates by a trapping procedure and the labelled compound incorporated into cholesterol, it is believed that the physical dispersion of the material in the medium is a limiting factor in this work. This is supported by the excellent results obtained when the squalene was incorporated into liver slices *in vivo* by prior feeding of the animals (11), and also by the fact that a vigorous agitation of the incubation mixture was found necessary for satisfactory results.

The inability of "starved" homogenates to incorporate squalene into cholesterol suggests a block in this portion of the cholesterogenesis pathway. In addition the failure of "starved" homogenates to incorporate acetate into squalene suggests an additional block between HMG or a near homologue and squalene.

Isolation of fractions from large scale incubations and examination of these with respect to their C^{14} content revealed that only in the residual aqueous phase was activity left unaccounted for, comparable to what would be expected to appear in cholesterol if there was no metabolic block in "starved" homogenates. In the other fractions the activity has been accounted for by the incorporation of 1-C^{14}-acetate into cholesterol, fatty acids, or compound *x*. Continuous liquid–liquid extraction of the residual phase with diethyl ether has shown that much of this residual activity can also be accounted for as compound *x*. The appearance of any other major radioactive component thus seems unlikely.

The incorporation of *x* into cholesterol by "normal" but not by "starved" homogenates places this compound before the metabolic starvation block.

The fact that HMG reduces its incorporation into cholesterol in "normal" homogenates would suggest that it occurs close to HMG in the chain of events if HMG is a direct intermediate. The fact that it accumulates would tend to put it nearer to the natural block of "starved" homogenates rather than away from it.

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THE ROLE OF THE RETICULOENDOTHELIAL SYSTEM IN THE DISAPPEARANCE PATTERN OF EVAN'S BLUE DYE (T-1824) FROM CIRCULATING PLASMA[1]

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Abstract

The disappearance of Evan's blue dye (T-1824) from plasma of dogs and rabbits has been followed for 4 hours in normal and T-1824 pretreated animals. One group of rabbits was pretreated with Thorotrast and the T-1824 disappearance studied for 1 hour. In both species of animals the disappearance pattern consisted of an early rapid fall in plasma dye concentration (Phase I) which was followed by a slower disappearance rate (Phase II). Phase I lasted approximately 30 minutes in dogs and slightly longer in rabbits and was curvilinear on a semilogarithmic plot. The disappearance rate during this period was markedly reduced by reticuloendothelial system (RES) blockade induced by pretreatment with T-1824 or Thorotrast. Phase II was characterized by a linear disappearance rate on semilogarithmic plot. It was more rapid in rabbits than in dogs and was unaffected in either species by RES blocking agents. The findings suggested that the RES was involved only in the first phase of T-1824 removal from plasma and that T-1824 was as effective as Thorotrast in blocking granulopexic activity of the RES.

The disappearance of Evan's blue dye (T-1824) from circulating plasma has been described as being linear (7), semilogarithmic (6, 10, 14), or divided into two or more phases (4, 5, 8, 11, 12, 13). Its loss from the plasma has been attributed to binding and filtration with plasma proteins (3, 6, 10) or, at least in the early phase, to reticuloendothelial granulopexic activity (4, 8, 13). The present investigation was carried out to compare the dye-disappearance pattern from plasma in dogs and rabbits over a 4 hour period and to study the effects of altering reticuloendothelial activity on the disappearance of T-1824 from the circulation.

Methods

Dogs

Dogs weighing 6–10 kg. were used in this study. Under local procaine infiltration venipuncture was performed with a 17 gauge needle, and a small polyethylene catheter was inserted into the antecubital vein and advanced to a depth of 8–10 cm. The needle was removed and the catheter secured with adhesive tape. The indwelling catheter permitted repetitive sampling of freely flowing blood at precise sampling times and reduced the problem of hemolysis. Blood samples were withdrawn into heparinized syringes at 5, 10, 20, 30, 60, 120, 180, and 240 minutes after the injection of the T-1824. The dye was dissolved in normal saline and the concentration adjusted so that in all cases the volume injected was 1 ml./kg. body weight. Eleven

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studies were carried out on normal dogs to establish the normal disappearance pattern. Five of the animals received 2.5 mg./kg. and the dose for the other dogs ranged from 0.8–3.0 mg./kg. with a mean of 1.95 mg./kg. for the group. Seven experiments were carried out on dogs which on the previous day had received intravenous injections of T-1824. Three of the dogs in this group received 2.5 mg./kg. The dose injected in the other animals ranged from 1.2–3.0 mg./kg. with a mean of 2.1 mg./kg. for the group. On the day of the experiment the animals were given the same amount of the dye and its disappearance pattern was studied in the usual manner.

Rabbits

The experiments with rabbits were carried out on animals ranging in weight from 2.0 to 4.5 kg. Indwelling polyethylene catheters were inserted into either the marginal or central veins of the ear and advanced toward the heart. Maximal venous dilatation was achieved by applying hot compresses to both ears (the compress applied to the contralateral ear during venipuncture maintained vasodilatation by reflex action). The same sampling procedure was used as that given above for dogs following the intravenous injection of 1 mg./kg. T-1824 in saline, made up to a concentration of 1 mg./ml. The T-1824 disappearance pattern was studied in a series of 15 animals to establish the normal pattern for rabbits. Three rabbits were pretreated with T-1824 (1 mg./kg. intravenously) on the previous day and four rabbits were pretreated with Thorotrast (24% to 26% stabilized colloidal thorium dioxide; 25% aqueous dextrin; 0.15% methyl parosept as preservative) at a dose range of 1–3 mg./kg. intravenously either 5 or 6 hours prior to determination of the dye disappearance pattern. In the rabbits receiving Thorotrast the disappearance pattern was studied for only 1 hour after the dye was injected.

T-1824 Determination

The blood samples (1 ml.) were centrifuged immediately after they were obtained and the plasma was removed. After dilution of the samples with 0.9% NaCl, the optical density of the T-1824 was determined in a Coleman Junior Spectrophotometer and compared with a normal curve prepared from T-1824 in phosphate buffer. To determine quantitatively the optical density of T-1824 in the presence of hemolysis, the O.D. of the solution was determined at 410 and 610 mμ and the following formula applied:

$1.006 - 0.045\ (D_{410}/D_{610}) \times 100 = \%\ D_{610}$ due to T-1824, where D = O.D. at the designated wavelength. The formula was derived in the following manner:

1. For T-1824, $(D_{410}/D_{610}) = 0.1367$.
2. For oxyHb, $(D_{410}/D_{610}) = 22.356$.
3. For an unknown mixture of T-1824 and oxyHb the contribution of T-1824 to the ratio can be calculated:
   $(D_{410}/D_{610}) = 0.1367$ T-1824 $-$ 22.356 Hb.
4. The proportion of the O.D. due to T-1824 in any mixture of T-1824 and oxyHb is T-1824 = 1.0 $-$ Hb.

5. By solving the equations simultaneously, the proportion of the D_{610} due to T-1824 is $1.006 - 0.045\ (D_{410}/D_{610})$.

Because no significant difference could be determined for (D_{410}/D_{610}) for oxyHb from rabbits and dogs, the same formula was used to correct for hemolysis in samples from both species.

Results

In rabbits and dogs there were two distinct phases in the rate of dye disappearance from the plasma (Fig. 1). Phase I was characterized by a curvilinear fall in the plasma concentration of the dye on a semilogarithmic plot. It lasted for about 30 minutes in dogs and slightly longer in rabbits. Phase II showed a slower drop in the plasma concentration of the dye and it appeared to be linear on a semilogarithmic plot for at least 3–3½ hours. When the reticuloendothelial system (RES) was blocked by pretreatment with either T-1824 (dogs and rabbits) or Thorotrast (rabbits) there was a slower fall in plasma dye concentration in phase I but phase II was unaffected by T-1824 pretreatment. Although the dye loss in phase I was markedly reduced by such pretreatment, it was still characterized by a more rapid

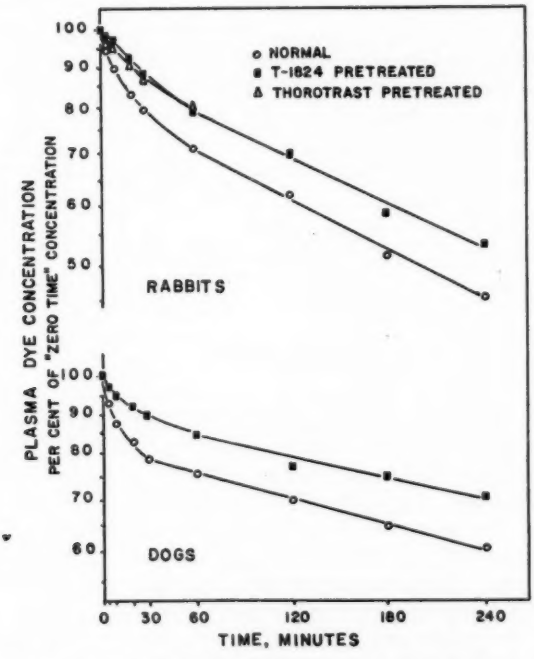

FIG. 1. Disappearance pattern of T-1824 from plasma of rabbits and dogs during a 4 hour period. Expressed as % of dye remaining in the circulation.

rate of dye loss than that in phase II (see Fig. 1 and Table I). The difference between normal and T-1824 pretreated dogs demonstrated a Student '*t*' value of 7.5 for phase I and 0.3 for phase II. Phase I showed a significantly greater dye-disappearance rate than phase II in all groups. The Student '*t*' values for a difference between the two phases were: for normal dogs, 14.7; for normal rabbits, 6.6; and for dogs pretreated with T-1824, 15.0.

These observations are quantitated in Table I. The average disappearance rate of the dye from the plasma is expressed as % lost per minute during the two phases. For normal dogs and rabbits the disappearance rate was the same during phase I (0.70 in both) while it was much greater during phase II in rabbits (0.14) than in dogs (0.078). The effect of blocking the RES was limited to phase I and was of the same magnitude in dogs and rabbits. T-1824 was as effective as Thorotrast in reducing the dye-disappearance rate in the experiments with rabbits (see Table I). In the experiments with dogs the dose of T-1824 varied from 0.8 to 3.0 mg./kg. No difference was observed in the dye-disappearance rate in those animals receiving the low and high doses of T-1824. However, the number of animals was small at the extremes of the range, and the dose range was too limited for firm conclusions to be drawn from the data on this point.

TABLE I

MEAN VALUE AND STANDARD ERROR (STANDARD DEVIATION OF THE MEAN) FOR THE CIRCULATING PLASMA. EXPRESSED AS % LOST PER MINUTE

	Dogs		Rabbits		
	Normal	T-1824 pretreated	Normal	T-1824 pretreated	Thorotrast pretreated
No. animals	11	7	15	3	4
Phase I (0–30 min.)	0.70 ± 0.04	0.37 ± 0.02	0.70 ± 0.06	0.40	0.43
Phase II (60–240 min.)	0.078 ± 0.01	0.075 ± 0.01	0.14 ± 0.06	0.14	

Discussion

Phase I

Those investigators who recognized an early rapid disappearance of T-1824 from the plasma believed it was due to slow mixing of the dye with non-circulating plasma (5, 11, 12), to RES activity (8, 13), or to a combination of these two factors (4). Conclusions regarding the role of the RES in the dye removal can be drawn by studying the effects of altering RES activity. Thorotrast pretreatment has been shown to interfere with the removal of a variety of colloidal substances from the circulation (8). Paldino, Sosnow, and Hyman (13) demonstrated that it blocked the RES but had no effect on the transcapillary transfer of T-1824. Although the literature is controversial regarding the ability of T-1824 to block the granulopexic activity of the RES (2, 4, 11, 12, 15), the results from the present study demonstrated a

decrease in phase I in dogs and rabbits, and in rabbits the magnitude of the decrease was the same as that produced by Thorotrast. The observation that phase II was not altered by T-1824 suggested that it did not alter the transcapillary loss of the dye.

Although the rate of dye removal was reduced markedly by procedures which blocked the RES, the drop in plasma concentration of the dye during phase I was still greater than that seen in phase II. This indicated that there was either not a complete block of the granulopexic activity of RES or that some factor in addition to the RES contributed to the accelerated rate of dye removal in phase I. Slow mixing of the dye with non-circulating plasma may have contributed to the accelerated drop of plasma T-1824 concentration. If a decrease in RES activity resulted from T-1824 injection (as indicated from the present work) a gradual decrease in the rate of dye removal would be expected in this phase. The same pattern would also be expected if there was a slow mixing period which lasted during the same phase. These factors, either alone or combined, may have been responsible for the curvilinear response seen in phase I. Slow mixing of the dye with non-circulating plasma may be responsible for the more rapid disappearance observed in phase I than in phase II after the RES has been blocked, although no evidence for this was obtained in the present study.

Phase II

The second phase in the disappearance curve of T-1824 from the plasma was linear on a semilogarithmic plot. It was at a faster rate in rabbits than in dogs and was not altered by pretreatment with T-1824 in either species. It was concluded that the RES was without effect on phase II of the disappearance pattern. Much evidence has supported the concept of a loss of T-1824 from the plasma by transcapillary transfer of protein tagged with the dye (1, 3, 6, 7, 9, 14, 15). However, the ultimate fate of T-1824 in the body would affect the pattern of dye disappearance, particularly in phase II. It would be expected that with the transcapillary loss of the dye, the dye-transfer theory, or any other mechanism which would come to an equilibrium with the T-1824 distributed throughout the body, the disappearance pattern would be curvilinear in phase II. At least during the 4 hours of studying these experiments, the loss appeared to be linear in this phase. Clausen and Lifson (1) attributed the loss of dye from the body to decolorization since dye loss through excretory pathways is not significant (1, 11). The fate of T-1824 in the body has an obvious bearing on the pattern of its disappearance from the plasma and is the subject of continued investigation at the present time.

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ELECTROLYTE METABOLISM IN THE RAT EXPOSED TO A LOW ENVIRONMENTAL TEMPERATURE[1]

D. G. Baker and E. A. Sellers

Abstract

Some aspects of electrolyte metabolism in the rat exposed to a low environmental temperature have been examined. The first day of exposure at 2° C. was accompanied by a loss of chloride. Continued exposure to cold resulted in a retention of sodium, and to a lesser extent, of potassium. Exposure to cold for 45 days (at 2° C.) caused a sustained elevation of concentration of sodium in the plasma, with a transient increase in potassium and no change in the chloride concentration. Prolonged exposure to cold resulted in an increased blood volume and total body water content. These observations suggest that the rats acclimatized to cold have a larger proportion of actively metabolizing tissues than do comparable animals at room temperature.

It has been appreciated for some time that changes occur in the distribution of electrolytes in animals exposed to an environmental temperature below that to which they are accustomed. Exposure to cold under conditions that do not appreciably alter the deep body temperature of the animal results in hemoconcentration (4, 5), a decrease in whole blood chloride (14), a diuresis with increased sodium chloride excretion (11), and an increase in serum potassium (16). These responses, it is generally conceded, are transient in nature. Much less is known concerning more permanent changes in electrolyte metabolism that develop during acclimatization to cold. The object of the investigations reported in this paper was to study electrolyte metabolism during acclimatization to a low environmental temperature.

Materials and Methods

Female rats of the Wistar strain, bred in our own department, were used throughout this work. The rats were offered Fox Breeder Cubes (Master Feeds Ltd.) and tap water ad libitum. Animals all weighed between 175 and 200 g. at the start of the experiments.

Rats exposed to cold were kept in individual cages at 2° ± 2° C.

During balance studies the quantitative collection of excreta was accomplished by placing a Pyrex tray immediately under each metabolism cage. The total collection for each animal was homogenized, and aliquots were taken for the determination of sodium, potassium, and chloride. Collections were made daily and, at these times, body weight and food intake were measured.

Blood samples were taken by direct cardiac puncture without anaesthesia (or other treatment) (3).

Sodium and potassium were determined using a Beckman DU Spectrophotometer with flame attachment. Plasma chlorides were measured by the method of Schales and Schales (21) while the chlorides excreted were

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measured by a modification of the procedure described by Van Slyke (23). The hematocrit value, hemoglobin, and total plasma protein concentrations were estimated using the specific gravity method of Phillips *et al.* (18). Plasma volume was measured using rat plasma protein labelled with iodine-131 and applying a simple dilution principle. Total blood volume could then be calculated using the plasma volume and the hematocrit value. Total body water was determined by drying the carcass to constant weight at a temperature of 97° C.

Experiment 1.—Changes in the plasma sodium, potassium, and chloride concentrations and in hematocrit value, hemoglobin, and plasma protein concentrations were followed in rats during acclimatization to cold. One blood sample was taken from each of eight animals at 1, 3, 8, 16, 24, and 48 hours and at 4, 8, 21, and 45 days after exposure.

Experiment 2.—Blood volume and total body water were estimated in rats after brief exposure to cold (12 and 24 hours) and after acclimatization to cold (over 45 days). Since, under our experimental regimen, rats in the cold grow at a slower rate than those at room temperature (25° C.) both age and weight controls were necessary in order to study the effects of prolonged exposure.

Experiment 3.—Total electrolyte balances with respect to sodium, potassium, and chloride were followed in 12 rats. From the daily food intake and the electrolyte composition of the diet (determined by analysis), the amount of sodium, potassium, and chloride taken in were calculated. Analysis of the daily excreta (urine plus feces) indicated the amount of each electrolyte lost by the rat. The difference between the electrolytes taken in and the electrolytes excreted is referred to as the electrolyte balance. After a control period of 1 week during which the electrolyte balance at room temperature was observed, the rats were placed in the cold room and the balance followed for a further 2 weeks.

Results

Experiment 1 (Figs. 1 and 2)

There was a trend toward a permanent increase in the concentration of plasma sodium during the acclimatization period, although only at the 192 hour period was the sodium concentration significantly higher ($P < .05$) than the pre-exposure level. After about 10 days the concentration of sodium tended to return to the pre-exposure level. Plasma chloride concentrations were unaltered during the exposure to cold. The concentration of plasma potassium was found to be at a maximum value after 1 hour of exposure. Statistically significant increases in the potassium concentration were observed at the 1, 3, 16, and 192 hour periods ($P < .05$). The values then fell toward those of the pre-exposure period.

Changes in the hematocrit value, hemoglobin, and plasma protein concentrations showed that a transient hemoconcentration existed for only the first few hours of exposure.

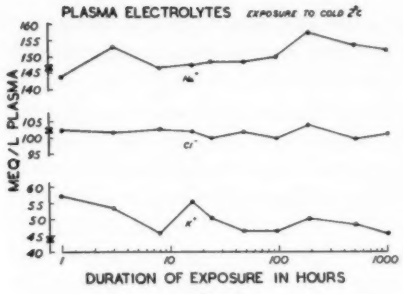


FIG. 1

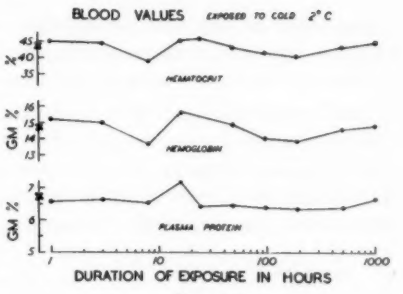


FIG. 2

FIGS. 1 and 2. Certain of the blood components during prolonged exposure to cold. Values from non-exposed (control) animals are indicated by the heavy ×. Note that the abscissa (exposure time in hours) is plotted on a logarithmic scale.

Experiment 2 (Table I)

No great volumetric changes occurred in body fluids within 24 hours after exposure to cold. A small decrease in total body water was observed after 12 hours. After prolonged exposure of the animals (45 days), the plasma volume, whole blood volume, and body water were significantly increased ($P < .05$) relative to volumes for controls of the same age, as were, also, plasma volume (expressed either as a percentage of body weight or of body water) and whole blood volume (expressed as a percentage of body weight) relative to those of controls of similar weight.

TABLE I

BODY FLUIDS DURING EXPOSURE TO COLD

Treatment	No. rats	Av. body wt., g.	Plasma vol., ml. per 100 g. b. wt.	Plasma vol., ml. per 100 g. b. wa.	Whole blood vol., ml. per 100 g. b. wt.	Whole blood vol., ml. per 100 g. b. wa.	Whole blood vol., ml. per 100 g. d. wt.	Body water, %
Controls for (A) and (B)	10	208	4.25	6.27	7.67	11.33	24.06	67.4
(A) 12 hours in cold	14	216	4.23	6.52	7.60	11.82	22.11	64.9
(B) 24 hours in cold	10	206	4.12	6.21	7.46	11.28	22.31	66.0
Age controls for (C)	12	276	3.86	6.39	7.11	11.74	18.03	59.7
Wt. controls for (C)	11	224	4.20	6.55	7.82	12.16	22.19	63.5
(C) 45 days in cold	14	225	4.75	7.34	8.63	13.36	24.58	64.6
						A.C.	A.C.	A.C.

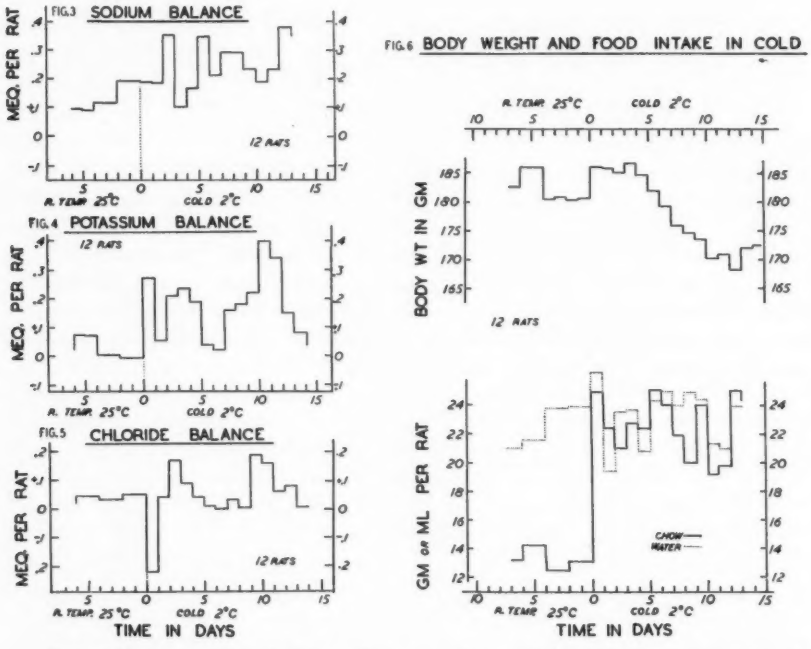
The plasma volume, blood volume, and body water values after exposure to cold. Results are expressed in terms of body weight (b. wt.), dry carcass weight (d. wt.), and body water (b. wa.). "Cold exposed" values which differ significantly ($P < .05$) from the corresponding control value are underlined. The letters A.C. in subscript indicate that the "treated" value differs significantly only from the age control.

Experiment 3 (Figs. 3, 4, 5, and 6)

The balance data indicated that exposure to cold results in retention of sodium and also a slight retention of potassium. During the first 24 hours of exposure there is a significant loss of chloride which was followed by a return to equilibrium, and this situation persisted throughout the exposure period.

Early in the exposure period the rats lost weight but, after approximately 11 days, this trend was reversed and an increase in weight occurred although at a rate somewhat less than observed at room temperature (25° C.).

The weight of food eaten increased immediately after exposure to cold. The food intake remained at about twice the value of that for rats of similar size at room temperature. Exposure to cold did not significantly affect the volume of water drunk by the rats although the total water intake (water drunk plus moisture of diet) was increased.



FIGS. 3, 4, and 5. The values for sodium, potassium, and chloride balance are expressed as the average number of meq. lost (−) or gained (+) per rat per day. This is indicated along the *Y* axis. The duration of exposure in days is shown along the *X* axis. Exposure to cold started on Day 0 and the periods of time before or after exposure are referred to the zero time. The length of each horizontal segment in the graphs gives the time period for which the indicated balance holds.

FIG. 6. The average food intake and body weights of the rats described in Experiment 3.

Discussion

During approximately 6 weeks of exposure to cold, the concentration of sodium in the plasma remained above that of the pre-exposure value. Barlow (6) found an increase in the sodium concentration in the plasma of troops who had been exposed to arctic conditions for 23 days. No change in the concentration of serum sodium or chloride was observed during 4 days when

Conley *et al.* (11) exposed humans to mild cold. Perhaps the discrepancy between these observations is partly due to the fact that Barlow's subjects and our rats had a greatly increased food intake and metabolic rate (22).

Plasma potassium concentration was increased from approximately 4.4 to 5.7 meq. per liter, after about 1 hour of exposure to cold. Increases in the concentration of plasma potassium have been described in animals subjected to acute exposure to cold (7, 21) and in men transferred from a warm to a cold environment (19). This could be related to the abrupt increase in metabolic rate which develops in animals exposed to cold or perhaps to a non-specific response to the cold stress.

At 12 and 24 hours after exposure to cold there was no significant change in blood volume. A small decrease in total body water was observed after 12 hours of exposure, possibly owing to a water loss through diuresis. These data are in agreement with the observations of Deb and Hart (12).

Pace and Rathbun (17) demonstrated that the water and nitrogen content of the body were relatively constant when expressed on a "fat-free" basis. Thus body water could be used to assess total body fat or lean body mass, the latter being an index of the amount of actively metabolizing tissue. Babineau *et al.* (1) have reported that the non-fat dry matter of rats is directly related to their total body water content and that prolonged exposure to cold has no effect on this relationship. Therefore, the increased content of body water of the rats exposed to cold was interpreted as indicating a larger proportion of actively metabolizing tissues in these animals. This conclusion is consistent with the well-known fact that the resting metabolic rate (MR 30°) (22) of rats acclimatized to cold is significantly elevated.

An increase of blood volume was observed in rats acclimatized to cold. The increased metabolic activity of these animals with a correspondingly increased oxygen requirement might well be the stimulus for the change in blood volume since the transient hemoconcentration seen when animals are first exposed to cold (12) increases the total oxygen-carrying capacity of the blood. Brown *et al.* (8, 9) have observed that both blood volume and metabolic rate of the Eskimo are elevated compared to values commonly found in the temperate zones and these values are minimal during the summer months.

During the period of exposure to cold a small retention of sodium was observed. The fate of this excess sodium is still obscure. It is possible that there is a shift of sodium (and perhaps water) into those superficial tissues that have temperatures somewhat less than that of the deeper body tissues. We expect that experiments recently completed will elucidate this problem.

In Table I it will be seen that the average value found for the total body water of "age controls" is lower than that of other control groups. These animals weighed considerably more than other controls or experimental groups. The smaller proportion of body water is consistent with the larger amount of body fat to be expected in animals of this weight. In other words, the higher

proportion of body water found in the acclimatized group of similar age reflects, to some extent, a difference in total body fat (12, 17*b*).

In the course of 15 days' exposure to cold three "peaks" of positive balance with respect to potassium were found. The physiological significance (if any) of the "peaks" is not apparent, but the potassium retention may be related to the accelerated energy metabolism of the rats exposed to cold (13).

On the first day of exposure to cold, a marked loss of chloride (negative balance) was observed. Other than this, the pattern of chloride balance follows closely those of other electrolytes. Other workers have reported an increased (2), decreased (10), or an unchanged (15) chloride excretion in animals exposed to cold. The loss of chloride on the first day of exposure to cold was of special interest to us. As a speculation, it is possible that the abrupt increase in food intake on Day 1 was accompanied by an increased gastric secretion of chloride, some of which could have been lost with the increased fecal excretion. Also, a significant increase in gastric acidity and chloride secretion has been reported in humans exposed to cold water (20) and in response to ACTH (24).

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THE SEPARATION OF OESTROGENS FROM AVIAN DROPPINGS[1]

R. O. HURST, A. KUKSIS,[2] AND J. F. BENDELL[3]

Abstract

Excretion of oestriol and oestrone by the hen and of oestriol, oestrone, and oestradiol by the rooster is indicated by chromatographic analyses and color tests on the neutral and phenolic steroid fractions of extracts of the droppings of sexually active domestic fowl. No evidence for the excretion of androgens was obtained. An unknown component of the neutral fraction reacted with ethanolic potassium hydroxide to produce a pink color and was found only in the extracts of droppings from the male fowl.

Introduction

In the course of a study of the abundance and local distribution of the ruffed grouse (*Bonasa umbellus* (L.)) it occurred to one of us (J. F. B.) that the droppings of grouse found in nature might contain sex hormones or their derivatives that would indicate sex and breeding condition. At the present time most of the information concerning the nature of the avian androgens and oestrogens has been deduced from the results of administering known mammalian sex hormones to birds. The precise nature of the sex hormones in birds has not been adequately demonstrated but previous reports by Gustavson (5) and by Riley and Fraps (7) have indicated the presence of oestrogenic material in the droppings of fowl.

Methods

Collection of Droppings

Two adult male and two adult female Plymouth Barred-Rock fowl were selected from penned flocks on the basis of good condition and productivity. The birds were maintained in separate compartments in a wire laying cage and fed on laying mash, oyster shell, and water. The droppings were collected from trays under the cages every 24 hours and were stored in separate cartons and kept at −40° C. until required.

Isolation of Phenolic and Neutral Steroid Fractions

The droppings were dispersed in 750 ml. of 1.7 *N* hydrochloric acid solution and the suspension was heated for 45 minutes at 100° C., cooled quickly to room temperature, and exhaustively extracted with carbon tetrachloride. The carbon tetrachloride was removed by evaporation to dryness *in vacuo* and the residue was taken up in toluene. The toluene solution was extracted with 1 *N* sodium hydroxide solution and the toluene phase set aside as the

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neutral steroid fraction. The alkaline phase was acidified with hydrochloric acid solution to pH 8.50 ± 0.02 and extracted with ether to obtain the phenolic steroid fraction. Both fractions were concentrated to dryness *in vacuo* and the residue taken up in a minimum volume of methanol. Samples of the methanol solutions were then taken for chromatographic analysis. This procedure isolated the fractions with the least contamination by the pigments present in the droppings since the pigments that were extracted by the carbon tetrachloride were found to be insoluble in ether. The phenolic steroid fraction was therefore obtained practically free of pigment. The pigments present in the neutral steroid fraction did not interfere with the chromatographic analysis since they were located either at the origin or at the solvent front.

Paper Chromatography

The procedure was essentially that described by Burton, Zaffaroni, and Keutman (3) as modified by Axelrod (1) for the determination of oestrogens and by Savard (8) for the determination of androgens. The solvent systems *o*-dichlorobenzene–formamide, and methylene chloride – formamide were used to analyze the phenolic steroid fraction. The method of Bush (4) was also employed for the analysis of oestrogens on paper impregnated with alumina in a system of 1:1 chloroform–benzene. The solvent system for the chromatography of the neutral steroid fraction was ligroin – propylene glycol (8).

Oestrogens were located on the chromatograms by immersion of portions of the strips in fuming sulphuric acid as described by Axelrod (1) and similarly in the Kober reagent as modified by Brown (2) for oestriol using 2% hydroquinone in sulphuric acid. Oestrogens were also detected by the method of Mitchell and Davies (6), in which the reagent of Folin and Ciocalteu is employed. The iodine–starch method described by Bush (4) was used with chromatography on alumina paper. Androgens were located on the chromatograms by examination with ultraviolet light from a Mineralite lamp at 2537 Å and by the Zimmerman reaction as used by Savard (8).

TABLE I

DETECTION ON PAPER CHROMATOGRAMS OF COMPONENTS PRESENT IN THE PHENOLIC STEROID FRACTIONS FROM THE DROPPINGS OF THE HEN AND ROOSTER

	Hen		Rooster		
Color reagent	Oestriol	Oestrone	Oestriol	Oestradiol	Oestrone
Fuming sulphuric (1)	+	+	+	±*	+
Kober (hydroquinone) (2)	+	±	+	+	±
Folin-Ciocalteu (6)	+	+	+	+	+
Zimmerman (8)	−	+	−	−	+
Iodine–starch (4)	+	+	+	+	+

*± indicates an uncertain observation based on a faint color response.

Results

Two components were located on chromatograms of the phenolic steroid fraction of the droppings of the hen that corresponded in R_f values to oestriol and oestrone run as standards. When the unknown components were eluted from the paper and the corresponding standard oestrogen added to the sample the unknown migrated the same distance as the standard in both cases.

In the analysis of the phenolic steroid fraction of the droppings of the rooster three components were found that ran to areas on the chromatograms corresponding to standard oestriol, oestradiol, and oestrone. Because of the low amounts of material that ran to the oestradiol area it was not possible to elute and rerun this component with standard oestradiol.

The component corresponding to oestrone was purified several times by rerunning on paper. This chromatographically purified material gave a definite precipitate with digitonin.

The results of the color tests on paper chromatograms for the components of the phenolic steroid fractions obtained from the droppings of the hen and the rooster are summarized in Table I.

No areas that were Zimmerman positive could be detected on chromatograms of the neutral steroid fractions from the droppings of the hen and the rooster although the chromatography of a standard mixture of testosterone, androsterone, and $\triangle^4$-androstene-3,17-dione gave results comparable to those reported by Savard (8). In one experiment 15 mg. of androsterone was added to a 24 hour collection of the droppings of a rooster. After the complete procedure was carried out, the androsterone was recovered without loss as determined by ultraviolet light absorption at 2400 Å.

One component was found in readily detectable amounts in the neutral steroid fraction from the droppings of the rooster. This component migrated 22 cm. in a 16 hour development period in the ligroin – propylene glycol system and gave a bright pink color when a portion of the chromatogram was immersed in ethanolic potassium hydroxide solution. It did not give a positive Zimmerman test or produce a color with fuming sulphuric acid and gave no color with the Folin-Ciocalteu reagent. When eluted from the paper it showed no absorption of ultraviolet light in the range 2200 to 3000 Å.

Discussion

On the basis of chromatographic evidence and color tests, it is concluded that oestriol and oestrone are present in the droppings of the male and female domestic fowl. The formation of an insoluble digitonide suggests that the oestrone is the biologically active form. A difference in steroid excretion between male and female is indicated by the observation that a component corresponding to oestradiol in chromatographic behavior is present in low concentration in the droppings of the rooster.

No evidence was obtained for the presence of testosterone, androsterone, or $\triangle^4$-androstene-3,17-dione or other ketosteroids reacting with the Zimmerman reagent in the neutral steroid fraction from the droppings of either sex

of the domestic fowl. The failure to observe androgens is probably due to the low amounts of these compounds excreted since androsterone added to the droppings of the rooster was recovered without loss. The unknown component that was found only in the neutral fraction from the droppings of the rooster may provide an additional basis for the determination of sex.

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A COMPARISON OF THE EFFECTS OF PROXIMAL AND DISTAL TUBULAR DAMAGE ON THE ACTION OF DESOXYCORTICOSTERONE AND ALDOSTERONE[1]

T. F. NICHOLSON

With the technical assistance of C. H. DOWNS

Abstract

The proximal tubules of the left kidney in dogs were damaged by the injection of 0.5% racemic sodium tartrate into the left renal artery. In other experiments the distal tubules were damaged by the injection of 0.05% mercuric chloride up the left ureter. In animals with proximal tubular damage, intravenous infusions of desoxycorticosterone or aldosterone which produced a significant drop in sodium excretion from the normal kidney had no effect on the amount of sodium excreted by the damaged kidney. In animals with distal tubular damage the effect of these hormones on the damaged kidney was as great as on the normal kidney.

Introduction

It has been generally assumed that the fine regulation of sodium by the kidney occurs in the distal segments of the tubule and that it is in these areas that the salt retaining hormones act (7). However, Bartter (1), by his diagrams illustrating the action of aldosterone, although not in his text, suggests that this hormone acts on the proximal tubule. In the present communication evidence is presented to show that in the dog the main, if not the sole, site of action of aldosterone and desoxycorticosterone is in the proximal convoluted tubule.

Experimental

Female dogs weighing from 10 to 14 kg. fed on the stock laboratory diet and with water ad libitum were used. Proximal tubular nephrosis was produced by injecting 0.5% racemic sodium tartrate into the left renal artery and removing it from the left renal vein by the method reported previously (5). Distal tubular damage was produced by passing a polyethylene catheter attached to a reservoir containing 0.05% mercuric chloride solution up the ureter and into the pelvis of the left kidney of female dogs. The catheter was tied in firmly with a slip knot and the reservoir was raised 150 cm. above the level of the animal and left there for 5 minutes.* The catheter was then detached from the reservoir, the slip knot was removed, and the pelvis and ureter were washed free of the mercuric chloride with 10 ml. of 0.9% saline solution. Seventy-two hours after the injection the animals were anaesthetized with sodium pentobarbital, the ureters were catheterized, and an intravenous infusion of 0.17 ml./minute of 0.9% sodium chloride

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Contribution from the Department of Pathological Chemistry, University of Toronto, Toronto, Ontario. This work was supported in part by a grant from the National Research Council of Canada.

*Preliminary experiments using phenol red and india ink showed that this procedure forced the fluid into the distal convoluted tubules but not into loops of Henle.

was started. After a stabilizing period of 30 minutes, urine was collected from each ureter for a half hour control period. The infusion was then changed to one containing either 0.6 μg./ml. desoxycorticosterone or 0.012 μg./ml. aldosterone* in isotonic saline, administered at the same rate. Following a half hour stabilizing period urines were again collected for a 30 minute "DOC" or "aldosterone" period. Blood was taken from the femoral vein at the beginning and end of each period, heparinized, and centrifuged immediately, and the plasma removed for analysis.

Sodium and potassium were determined on the urine by flame photometry and their rates of excretion calculated in microequivalents per minute. Creatinine was determined on plasma and urine by the method of Bonsnes and Tausky (3) and the creatinine clearance estimated for each kidney.

At the end of the experiment the kidneys were removed from the anaesthetized animals and fixed and examined by the methods previously described (5).

Results

The histological changes in the proximal tubule resulting from the injection of sodium racemate have been described in an earlier communication (5).

The changes in the distal convoluted tubules following the ureteral injection of 0.05% mercuric chloride comprised slight swelling of the cells and blurring of their outlines, increased granulation with intensification of the acidophilic staining, marked variation in nuclear staining, and some desquamation of cellular material into the lumens. The collecting tubules showed some slight evidence of damage but the changes were not marked. The proximal convoluted tubules were unaffected.

In some animals there were evidences of previous renal lesions such as scarring or cellular infiltration. The results from such animals were not considered.

The creatinine clearances by the damaged kidneys were 50 to 60% of the normal in the same animal. As the reduction in clearances following tubular damage has been shown to be due to increased back diffusion through the nephrotic tubules (2, 5) the diminution in clearance may be considered a rough index of the amount of damage.

The effect of proximal tubular damage on the sodium retaining action of desoxycorticosterone and aldosterone is shown in Table I. The effect of distal tubular damage is given in Table II. In the doses used both hormones produced a definite reduction in the amount of sodium excreted by the normal kidney but, except for one instance where double the dose of DOC was used, neither aldosterone nor desoxycorticosterone had any effect on the sodium excretion from the kidneys with proximal tubular damage. Even in the experiment where double the dose of DOC was used the damaged kidney showed a much smaller proportionate reduction in sodium excretion than did the normal kidney. Distal tubular damage on the other hand had no detectable effect on the action of either of the corticoids.

*We are indebted to Dr. A. G. Gornall for the aldosterone which was prepared under his direction by the method of Gornall and Gwilliam (4).

TABLE I

THE EFFECT OF DAMAGE TO THE PROXIMAL TUBULE ON THE ACTION OF DESOXYCORTICOSTERONE AND ALDOSTERONE

Dog No.	Period	Sodium excretion (μeq./min.)		Clearance ratio, nephrotic / normal
		Normal kidney	Nephrotic kidney	
3	Control	11.9	4.1	0.55
	DOC*	7.2	3.7	
4	Control	7.0	18.9	0.59
	DOC*	5.7	27.3	
6	Control	19.3	4.1	0.51
	DOC*	14.2	4.3	
5	Control	32.2	54.4	0.56
	DOC†	6.9	25.4	
7	Control	5.7	4.8	0.52
	Aldosterone‡	2.7	5.1	
9	Control	9.4	6.1	0.60
	Aldosterone‡	6.0	6.3	
14	Control	9.5	3.6	0.54
	Aldosterone‡	6.5	3.7	
15	Control	12.6	26.5	0.58
	Aldosterone‡	11.3	26.9	
19	Control	9.1	3.9	0.56
	Aldosterone‡	3.3	5.5	

*0.17 ml./min. of 0.6 μg./ml. desoxycorticosterone infused for ½ hour before urine collection started and continued for the ½ hour urine collection period.
†Double amount of DOC infused.
‡0.17 ml./min. of 0.012 μg./ml. aldosterone infused for ½ hour before urine collection started and continued for the ½ hour urine collection period.

TABLE II

THE EFFECT OF DAMAGE TO THE DISTAL TUBULE ON THE ACTION OF DESOXYCORTICOSTERONE AND ALDOSTERONE

Dog No.	Normal period	Sodium (μeq./min.)		Clearance ratio, nephrotic / normal
		Normal kidney	Nephrotic kidney	
L15	Control	1.16	2.62	0.54
	DOC*	0.63	0.72	
L16	Control	1.78	3.30	0.58
	DOC*	0.56	0.52	
L18	Control	1.34	2.85	0.56
	DOC*	0.49	0.57	
L13	Control	1.8	3.6	0.57
	Aldosterone†	1.3	2.2	
L8	Control	6.6	8.1	0.53
	Aldosterone†	5.2	6.2	
L9	Control	3.0	15.1	0.60
	Aldosterone†	2.6	10.4	
L14	Control	2.6	5.1	0.55
	Aldosterone†	1.7	3.9	

*0.17 ml./min. of 0.6 μg./ml. desoxycorticosterone infused for ½ hour before urine collection started and continued for the ½ hour urine collection period.
†0.17 ml./min. of 0.012 μg./ml. desoxycorticosterone infused for ½ hour before urine collection started and continued for the ½ hour urine collection period.

Under our conditions neither desoxycorticosterone nor aldosterone changed the rate of potassium excretion. In most of the animals with proximal tubular damage less sodium was excreted by the nephrotic kidney than by the normal kidney (Table I). On the other hand, when the distal tubules were damaged, the nephrotic kidney excreted the larger amount of sodium (Table II).

Discussion

The finding that proximal tubular damage inhibits the renal action of aldosterone and desoxycorticosterone and that distal tubular damage has no such effect forces one to conclude that the facultative reabsorption of sodium under the control of the adrenal cortex takes place in the proximal tubules. It appears evident that this activity of the proximal tubules must play a significant role in regulating the sodium load reaching the distal tubules. It is quite possible, as suggested by Wesson *et al.* (8), that the latter have a maximum capacity for sodium reabsorption and that the amount of sodium in the urine depends upon the amount by which the sodium load delivered to them by the proximal tubules exceeds that maximum. However, our experiments seem to demonstrate quite clearly that the reabsorptive capacity of the distal tubules is not, as has been suggested (7), varied by the action of the salt-retaining hormones of the adrenal cortex.

The effect of the tubular damage, per se, on sodium excretion is quite compatible with these theories. When the proximal tubule is damaged the increased back diffusion of water and sodium which occurs could, when the filtered load of sodium was of average proportions, decrease the amount of sodium reaching the distal tubules by a significant amount and thus cause a decrease in urine sodium. On the other hand an equivalent percentile increase in back diffusion through the distal tubule would result in a much smaller absolute loss of sodium from the tubular urine, a loss which could be more than balanced by decreased active reabsorption of sodium. However, when the sodium intake is high, studies in progress in our laboratory indicate that with unilateral proximal tubular damage more sodium is excreted by the nephrotic kidney than by the normal kidney (6). It is possible that when the filtered load of sodium is high enough the decrease in the ability of the cells of the damaged proximal tubule to reabsorb sodium counterbalances the increased back diffusion and that the sodium load reaching the distal tubules of the damaged kidneys is greater than in the normal kidney.

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A POSSIBLE MECHANISM FOR FATTY ACID INHIBITION OF CHOLESTEROL SYNTHESIS[1]

J. D. Wood and B. B. Migicovsky

Abstract

Further investigations have been carried out on the fatty acid inhibition of cholesterol biosynthesis in rat liver homogenates. A correlation appears to exist between cholesterol inhibition and the elongation of the carbon chain of saturated fatty acids containing an even number of carbon atoms. Neither saturated nor unsaturated fatty acids interfere with the formation of acetyl CoA by liver homogenate. The stage where acetoacetate is formed from acetyl CoA is suggested as a possible site for inhibition of cholesterol synthesis by fatty acids.

Introduction

The addition of fatty acids is known to interfere with acetate metabolism in liver preparations. Avigan *et al.* (1) showed that fatty acids such as butyric and octanoic bring about an inhibition of acetoacetate synthesis from acetate. Results published previously (Wood and Migicovsky (15)) showed that both saturated and unsaturated fatty acids inhibit cholesterol synthesis from acetate. We demonstrated that the addition of the acids leads to an increase in the C^{14} found in the fatty acid fraction after incubation, but that this increase is small compared to the decrease in C^{14}-incorporation into cholesterol. Further investigations of this phenomenon are presented in this paper.

Materials and Methods

Homogenates were prepared from the livers of 120–130 g. rats by the method of Bucher (3) with the following adaptations. The liver was perfused with ice-cold 0.25 *M* sucrose prior to excision, and the nicotinamide and magnesium chloride were omitted from the buffer solution since they were added directly to the incubation flasks.

The incubations were carried out in a Dubnoff water bath under oxygen at 37° C. for a period of 2.5 hours. The incubation mixture, except where otherwise stated, consisted of 4.0 ml. liver homogenate, 2.6 mg. ATP, 7.6 mg. DPN, 16 micromoles (μM.) C^{14}-acetate counting 8×10^6 c.p.m., 1 μM. $MgCl_2$, 20 mg. nicotinamide, and water to give a final volume of 7.0 ml. The fatty acids were added as their potassium salts in aqueous solution in the amounts indicated in the figure and tables.

The C^{14}-acetate incorporation into cholesterol was measured by the method of Migicovsky (10).

The C^{14}-incorporation into fatty acids was not itself measured but instead the specific activity of the fatty acid carbon after saponification of the incubation medium. This specific activity could be compared for a series of samples

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if they contained equal volumes of the same liver preparation, because the amount of fatty acids after saponification would be the same in each sample. The amount of fatty acid added to the incubation medium was disregarded because it was negligible compared with the endogenous material. The specific activity measured is proportional to the amount of C^{14}-acetate incorporated into the fatty acids. Therefore the degree of incorporation, although not actually determined, can be compared for a series of samples.

The fatty acids were isolated by acidifying the aqueous solution from which the cholesterol had been extracted with 10 *N* HCl. The acidified samples were extracted with two 50 ml. portions of petrol ether. The combined extracts were washed three times with water and taken down to dryness on a steam bath.

The specific activity of the fatty acid carbon was estimated as follows. The sample was combusted using the reagents of Van Slyke *et al.* (14). The carbon dioxide evolved from the combustion was absorbed in sodium hydroxide and precipitated with barium chloride. The resulting precipitate of barium carbonate was plated on paper and the radioactivity measured in a gas flow counter. The barium carbonate was then dissolved in *N*/50 standard hydrochloric acid and back titrated to obtain the amount of barium hydroxide. The total carbon in the sample and the specific activity as c.p.m./mg. carbon were calculated from these values.

The specific activity of the carboxyl carbon was obtained in a similar manner. The fatty acid was decarboxylated using the method of Phares (11) except that the reaction mixture was cooled to 0° C. before the beginning of the reaction. The carbon dioxide evolved was treated as described above, and the specific activity of the carboxyl carbon was calculated.

The formation of hydroxamic acids was measured as follows: 3.0 ml. liver homogenate was incubated at 37° C. under oxygen for 2.5 hours with 2 μM. $MgCl_2$, 1.3 mg. ATP, 3.8 mg. DPN, 10 mg. nicotinamide, 2 millimoles (mM.) hydroxylamine, 50 μM. C^{14}-acetate, and fatty acid as indicated in the tables. Water was added to bring the total volume to 4.5 ml. The quantitative determination of the water-soluble hydroxamic acids was made according to Kornberg and Pricer (8). The volumes used were double those recommended by Kornberg and Pricer because of the large volume of the sample being estimated. Acethydroxamic acid prepared by the method of Lipmann and Tuttle (9) was employed as a standard.

The water-soluble hydroxamic acids formed during the incubation were identified by paper chromatography. The incubation mixtures were centrifuged at 80,000 × *g* for 30 minutes and the clear supernate was decanted and freeze-dried. The dry powder was shaken with 10 ml. of absolute ethanol to extract the hydroxamic acids, and the mixture was centrifuged at 900 × *g* for 5 minutes. The clear solution so obtained was spotted on Whatman No. 1 chromatographic paper. The chromatogram was developed with *n*-butanol:acetic acid:water, (4:1:5 v/v). The chromatograms were dried at room temperature and sprayed with a solution containing 50 g. $FeCl_3.6H_2O$

in 1 liter of 95% ethanol containing 0.1 M. HCl. The hydroxamic acid spots appeared reddish on a yellow background. The R_f values were 0.53 for acethydroxamic, 0.75 for butyrhydroxamic, and 0.90 for longer chain acid derivatives.

Fatty acid chromatograms were run on Whatman No. 1 paper which had been pretreated with liquid paraffin as described by Spiteri (12), whose method for the color development was also employed. The solvent used was glacial acetic acid:water (25:1 v/v), which gave R_f values of 0.50, 0.62, 0.78, and 0.84 for stearic, palmitic, myristic, and lauric acids respectively. Shorter chain fatty acids moved with the solvent front.

Results

The effect of fatty acids on C^{14}-incorporation into cholesterol, into total carbon of the fatty acids, and into the carboxyl carbon of the fatty acids is shown in Table I. The percentage change in the different incorporations due to the added fatty acid was calculated from the data in Table I and plotted against the chain length of the fatty acid. The curves obtained are shown in Fig. 1. A striking similarity was observed between the curves for the inhibition of incorporation into cholesterol and the promotion of incorporation into the carboxyl carbon. The inhibition and promotion respectively increased with the carbon chain length to reach a maximum at 12 carbons. Further lengthening of the chain decreased both effects. No such similarity was observed with the incorporation into the fatty acid total carbon.

TABLE I

EFFECT OF FATTY ACIDS OF VARYING CHAIN LENGTH ON THE C^{14}-INCORPORATION INTO DIFFERENT GROUPINGS

The aliquots employed in the estimations of the specific activities of the total carbon and carboxyl carbon were $\frac{1}{10}$ and $\frac{1}{2}$ of the total sample respectively. The resulting barium carbonate precipitates had weights averaging 15 mg. and 5 mg. respectively.

Addition to incubation medium	C^{14}-acetate incorporated into cholesterol (μM. $\times$ 10^3)	Specific activity of fatty acid total carbon* (c.p.m./mg. carbon)	Specific activity fatty acid carboxyl carbon* (c.p.m./mg. carbon)
—	444.32	6,768	7,503
4.0 μM. caprylic	323.04	7,101	11,629
4.0 μM. capric	250.18	7,260	21,232
4.0 μM. lauric	189.58	7,582	25,566
4.0 μM. myristic	342.21	8,466	17,742
4.0 μM. palmitic	404.01	8,789	9,872

*In order to convert to the approximate C^{14}-acetate incorporations as μM. $\times$ 10^3 the values in the table are multiplied by 0.0175 and 0.0012 for the total carbon and carboxyl carbon respectively. The values so obtained are approximate since the amounts of total carbon and carboxyl carbon in the samples were not known exactly because of incomplete recovery of fatty acids during extraction.

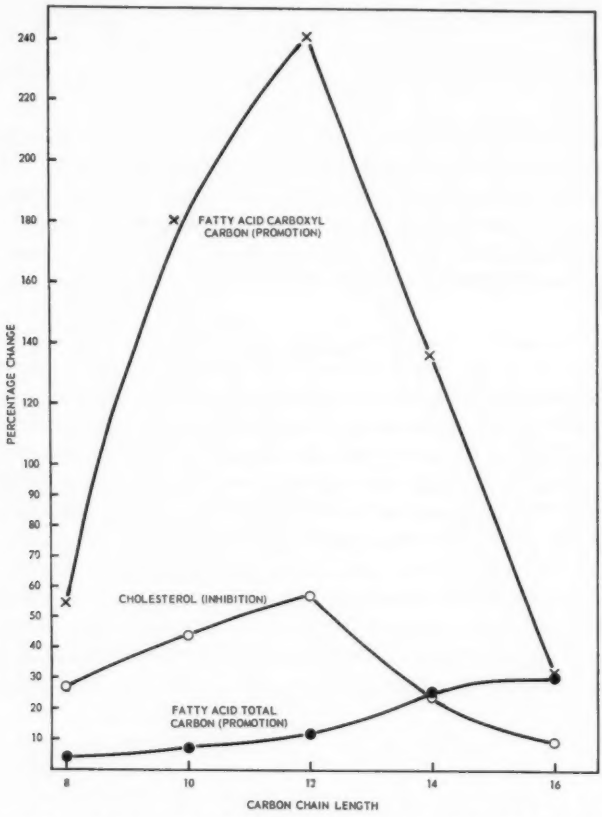

FIG. 1. Effect of fatty acids of varying chain length on the C^{14}-incorporation into different groupings (4.0 μM. fatty acid).

Chromatograms were run on duplicate samples of the fatty acid fraction after incubation, along with fatty acid controls. The controls and one set of samples were sprayed with alcoholic ferric chloride as described under methods. The positions of the fatty acids on the chromatogram for the remaining set of samples could therefore be located without prior color development, which eliminated losses in the fatty acids that might occur during development of the color. The areas containing fatty acids were cut out and the radioactivity measured. Table II shows the activity of the individual fatty acids isolated on the chromatogram after the samples had been incubated with and without added fatty acid. The results show that the presence of lauric acid decreased the amount of C^{14} found in fatty acids with a shorter carbon chain than lauric acid, but increased the incorporation into acids with a longer chain.

TABLE II

THE ACTIVITY OF THE INDIVIDUAL FATTY ACIDS ISOLATED BY PAPER CHROMATOGRAPHY

The amount applied to the chromatogram was equivalent to 1/25 of the total sample. Background count of 80 (±4) c.p.m. has been deducted from all figures

Area on the chromatogram corresponding to:	Addition to incubation mixture	
	No lauric acid	2.0 μM. lauric acid
10 carbon and shorter chain acids	50 c.p.m.	30 c.p.m.
Lauric acid	27	30
Myristic acid	49	284
Palmitic acid	18	26
Stearic acid	8	7

The effect of different types of fatty acids on the incorporation of C^{14} into various fractions is shown in Table III. The saturated fatty acids with an even number of carbon atoms are represented by lauric, the saturated acids with an odd number of carbon atoms by heptanoic, and the unsaturated acids by nervonic. The table also includes the effects of different concentrations of fatty acid. It is observed that an increase in the amount of lauric acid added to the incubation medium decreased the C^{14}-incorporation into cholesterol and increased the incorporation into the carboxyl carbon of fatty acids. On the other hand heptanoic acid or nervonic acid decreased both incorporations. In the latter two cases the decrease in the incorporation into carboxyl carbon paralleled the decrease of incorporation into the total carbon.

The formation of CoA derivatives by liver homogenates with and without added fatty acid was investigated. It was shown by Chou and Lipmann (4) that hydroxylamine, if present in high concentrations (0.5 to 1.0 molar), reacted non-enzymatically with acetyl CoA and other carboxyl-activated substrates to form the corresponding hydroxamic acids. The formation of the latter compounds was therefore used as an indication of the formation of the fatty acid CoA derivates. Incubation mixtures were prepared as mentioned previously, and duplicate samples of each were prepared but without the addition of hydroxylamine. The effect of fatty acids on the formation of water-soluble hydroxamic acid in the first set was compared with the effect on cholesterol synthesis in the second set. The results are presented in Table IV. They show that lauric and erucic acids, although inhibiting cholesterol synthesis, had little or no effect on the formation of the water-soluble hydroxamic acids and therefore on the corresponding fatty acyl CoA formation.

Chromatography of the water-soluble hydroxamic acids showed that acethydroxamic acid was the major constituent, with smaller amounts of butyrhydroxamic acid and only very small amounts of longer chain fatty acid derivatives. The presence of lauric acid and erucic acid during the

TABLE III

THE CHANGE IN THE C^{14}-INCORPORATION INTO VARIOUS GROUPS DUE TO DIFFERENT FATTY ACIDS AND DIFFERENT CONCENTRATIONS OF THE SAME FATTY ACID

Expt. No.	Addition to incubation medium	C^{14}-Acetate incorporated into cholesterol, $\mu M. \times 10^3$	Specific activity of fatty acid carboxyl carbon, c.p.m./mg. C	Specific activity of fatty acid total carbon, c.p.m./mg. C	Percentage change in C^{14}-incorporation due to the added fatty acid		
					Cholesterol	Fatty acid carboxyl carbon	Fatty acid total carbon
1	—	15.31	8,301				
	0.4 μM. lauric	11.06	16,957		−26	+104	
	1.0	6.30	25,297		−58	+204	
	2.5	2.10	68,003		−86	+719	
	3.0	1.51	77,543		−90	+834	
2	—	58.02	7,867	2,470			
	0.5 μM. nervonic	55.09	5,864	1,597	− 9	− 26	−35
	1.0	23.67	4,729	1,344	−62	− 40	−46
	1.5	0.46	1,155	99	−99	− 85	−96
3	—	44.07	11,489	4,604			
	4.0 μM. heptanoic	29.69	9,224	3,578	−33	− 20	−22
4	—	31.66	4,471	1,638			
	4.0 μM. lauric	0.59	27,151	2,618	−98	+507	+60

incubation did not visibly alter the number or the size of the spots on the chromatogram. This was in agreement with the quantitative studies mentioned above. It can be concluded therefore that fatty acids do not interfere with formation of fatty acyl CoA derivates and more particularly with formation of acetyl CoA. The fact that lauric and erucic acids did not alter the chromatographic picture shows that any hydroxamic acid derivatives of these acids formed during the incubation were not water-soluble to any significant extent, and therefore did not interfere with the quantitative studies described previously.

TABLE IV

EFFECT OF LAURIC AND ERUCIC ACIDS ON THE FORMATION OF CHOLESTEROL AND WATER SOLUBLE HYDROXAMIC ACIDS

The amounts of hydroxamic acids formed at zero time varied from 0.20 to 0.29 μM. and have been subtracted from the appropriate values in the table

Experiment number	Addition to incubation medium	C^{14}-acetate incorporated into cholesterol, μM. × 10^3	Hydroxamic acids formed, μM.	Percentage changes due to the added fatty acids	
				Cholesterol	Hydroxamic acids
1	—	70.90	3.61		
	1.0 μM. lauric	56.77	3.61	−19.9	0.0
	1.0 μM. erucic	61.92	3.65	−12.7	+1.1
2	—	39.30	3.91		
	1.0 μM. lauric	31.50	3.81	−19.8	−2.5
	1.0 μM. erucic	33.32	3.91	−10.1	0.0
3	—	11.10	1.87		
	1.0 μM. lauric	8.38	1.87	−24.5	0.0
	1.0 μM. erucic	9.21	1.89	−17.0	+1.1

Discussion

The presence of a saturated fatty acid, containing an even number of carbon atoms, during incubation of C^{14}-acetate with rat liver homogenate produced a decrease in the C^{14}-incorporation into cholesterol, and a large increase in the amount of C^{14} entering the carboxyl group of fatty acids. The percentage increase in the incorporation of C^{14} into the total carbon was only a fraction of the percentage increase into the carboxyl carbon. Now the chromatographic results reported here show that the increase in the C^{14} in fatty acids was located in the acid with two more carbons in the chain than the lauric acid added to the incubate. These facts taken together indicate that C^{14}-acetate is being added on to the fatty acid in the incubate to form the acids with longer chains. This is a synthesis which was shown to occur in rat tissue preparations by Tietz and Popjak (13), and which is analogous to the reversal of β-oxidation. It involves a series of reactions, (Green (6)), which can be summarized as follows:

(1) fatty $acid_{2n}$ + CoA ⇋ fatty $acid_{2n}$ CoA,

(2) fatty $acid_{2n}$ CoA + acetyl CoA ⇋ fatty $acid_{2n+2}$ CoA + CoA, where $2n$ is the number of carbon atoms in the molecule.

The increase in the C^{14}-incorporation into carboxyl carbon can therefore be taken as a measure of the elongation of the fatty acids. This reaction was shown to bear a close relationship to the inhibition of C^{14}-incorporation

into cholesterol over a series of saturated acids and over a series of concentrations of the same acid. This suggests the possibility that the reaction involving the elongation of the fatty acid chain may be the cause of the inhibition of cholesterol synthesis.

The question now arises as to the possible site of the inhibition. One possibility is that the fatty acid and C^{14}-acetate are competing with each other in reaction 1. However, the experiments detailed in this paper show that the addition of fatty acid to the incubation medium exerts no influence on the formation of acetyl CoA by the liver homogenate.

Another possible mode of inhibition is the combination of C^{14}-acetate with the fatty acid by reaction 2, thereby reducing the amount of C^{14}-acetate available for cholesterol synthesis. However it was shown previously (Wood and Migicovsky (15)) that the increase in the C^{14} incorporated into the fatty acids was much less than the decrease into cholesterol and an increase in C^{14}-acetate did not overcome the inhibition. This would indicate that the withdrawal of available C^{14}-acetate is not responsible for the inhibition of cholesterol synthesis. Neither is the inhibition due to a withdrawal of available CoA because addition of that compound increases the inhibition (Wood and Migicovsky (15)).

There remains the possibility that some part of the enzyme system in reaction 2 is also employed in the cholesterol synthesizing system, and that addition of fatty acid to the incubate diverts the enzyme system from cholesterol synthesis to fatty acid elongation. This possibility is supported by data presented by Wood and Migicovsky (15), who showed that inhibition of cholesterol synthesis by a given quantity of fatty acid was decreased in the presence of larger quantities of homogenate.

Now acetoacetate is a precursor of cholesterol synthesis according to Brady and Gurin (2) and Curran (5). Jowett and Quastel (7) demonstrated its formation from acetate. It is therefore strongly suspected of being an intermediate in cholesterol synthesis. It is formed by the condensation of two molecules of acetyl CoA, which is just a particular instance of the formation of a β-keto acid in the reactions making up the system used for the elongation of the fatty acid chain. Here, then, is an instance of a reaction which is common to cholesterol synthesis and elongation of the fatty acid carbon chain, and which may be the site of the inhibition of cholesterol synthesis.

The fact that long chain unsaturated fatty acids and odd numbered carbon chain saturated acids do not show an increase in carboxyl C^{14}-incorporation does not necessarily negate the above conclusions. The fatty acyl CoA derivative may still combine with a factor employed in the "common" reaction to form a complex inactive with respect to fatty acid elongation. In this case cholesterol inhibition could still occur, but no C^{14}-acetate would be added on to the fatty acid and therefore no C^{14} would be found in the carboxyl carbon.

If the mode of inhibition is as postulated, then fatty acids should inhibit acetoacetate formation from C^{14}-acetate and also the *de novo* synthesis of fatty acids from C^{14}-acetate. Avigan, *et al.* (1) showed that acetoacetate formation was in fact inhibited by fatty acids. The *de novo* synthesis of fatty acids cannot be measured when a saturated fatty acid with an even number of carbons has been added to the incubation mixture, because the elongation of the fatty acid interferes. However, this difficulty is not encountered with the other types of fatty acids for no elongation occurs. In this case the C^{14}-incorporation into the total carbon is a measure of the *de novo* synthesis of fatty acids from C^{14}-acetate, and this incorporation was shown to decrease with increase in the amount of added fatty acid, i.e. fatty acids inhibit the *de novo* synthesis.

The results presented here therefore suggest the possibility that fatty acids inhibit cholesterol synthesis at the step where acetoacetate is formed from acetyl CoA, and do so by combining with a factor in the enzyme system to form a complex. This complex is involved in the elongation of the carbon chain when the fatty acids are saturated and contain an even number of carbon atoms, but it has no such synthetic role when the acids are unsaturated or contain an odd number of carbon atoms in the chain.

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BIOCHEMICAL PROPERTIES OF PENTASERINE (α-AMINO-δ-HYDROXYVALERIC ACID)

1. NON-AVAILABILITY OF ARGININE FROM PENTASERINE IN THE GROWING RAT[1]

FERNAND MARTEL

Abstract

Pentaserine was fed at different levels to rats on an arginine-deficient diet and was shown to have no growth-promoting effect. This is at variance with what has been obtained in chickens where pentaserine could replace arginine partially in supporting growth.

In 1951 (3) we indicated the DL-α-amino-δ-hydroxyvaleric acid (pentaserine) could partially replace arginine in the growing chicken, and suggested that this substance was utilized in the *in vivo* synthesis of arginine.

This was of a particular biochemical interest since ornithine (α,δ-diaminovaleric acid), which is chemically closely related to pentaserine, had already been shown to be ineffective in such instances (2).

The present work was undertaken to gain additional evidence that pentaserine can take part in the *in vivo* synthesis of arginine by testing the possibility of a growth promoting effect in arginine-deficient rats.

Experimental

Young albino male rats of the Wistar strain were given access to a basal, arginine-free diet similar in composition to that used by Rose *et al.* (1) in determining the indispensability of arginine. They were divided into three groups of 10 animals, as homogeneous as possible with respect to body weight distribution. Group I received the deficient diet alone, group II the same diet plus 0.24% of L-arginine hydrochloride, and group III the basal diet with 0.24% DL-pentaserine added. Supplementations were made at the expense of sucrose. Animals were housed separately and allowed to ingest the diets ad libitum.

From the seventh up to the 14th day of the 23 day experimental period, group III was subdivided and the diet modified so as to include pentaserine at three different levels: 0.5% (group III*a*, three rats), 1.0% (group III*b*, four rats), and 2.0% (group III*c*, three rats). For the last nine days of the experiment, the initial group III was reformed and given pentaserine at the level of 0.5% (group III*d*).

Animals were weighed every 2 or 3 days, and growth curves recorded. Food consumption was measured every day.

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Contribution from the Department of Biochemistry, School of Medicine, Laval University, Quebec, Que.

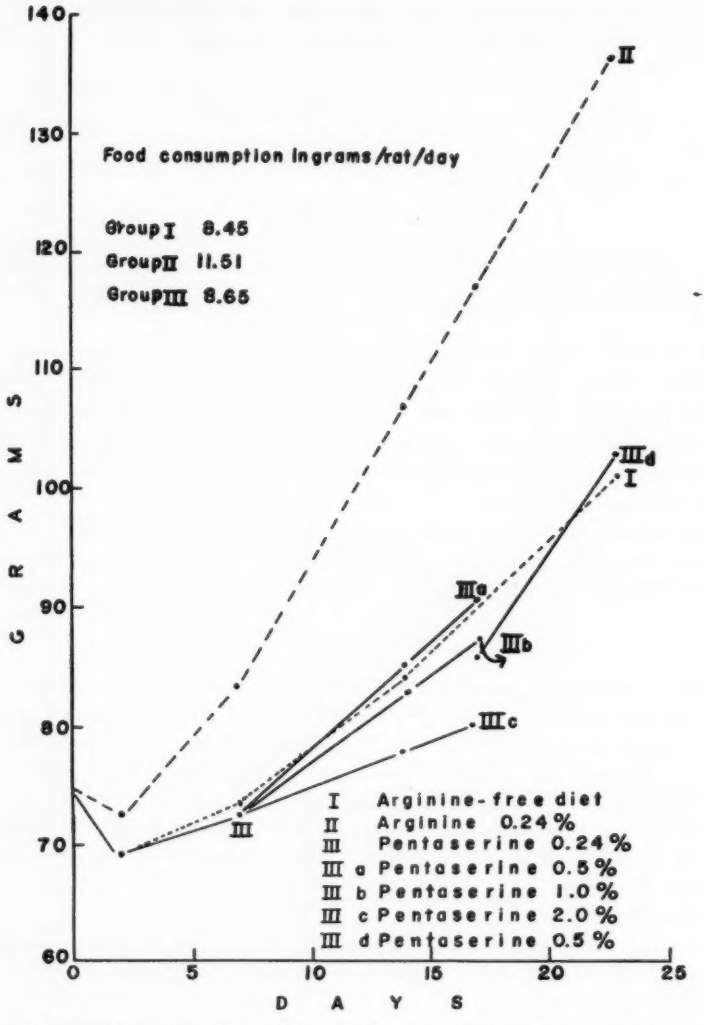

FIG. 1. Growth curves of arginine-deficient rats fed on diets supplemented with arginine or pentaserine.

Results

The growth curves of Fig. 1 show that the basal diet alone (group I) is ineffective in eliciting a satisfactory growth response in young rats, but that the addition of 0.24% of arginine is highly beneficial (group II) as could be expected from the results of Rose *et al.* (1).

The inclusion of 0.24% of pentaserine in the deficient ration adds nothing more to the growth response than does the basal diet alone, and seems even slightly detrimental. Pentaserine given at 1.0% (group III*b*) and 2.0% (group III*c*) acts as a growth retarder in the deficient animals, while at 0.5% (group III*a* and III*d*) it produces nothing but a very slight and probably insignificant positive response.

Mean daily food consumption, per rat, is higher in the arginine-fed rats than in the two other groups (Fig. 1).

Conclusions

These results indicate that no arginine can be made available from pentaserine for growth purposes in the rat, and are at variance with results obtained in the chicken (3).

This contradiction does not exclude the possibility of arginine synthesis from pentaserine in the rat, the newly formed amino acid being possibly utilized in this species for purposes other than growth-promotion.

It is unlikely that pentaserine exerts any peculiar effect on appetite since food consumption is not significantly modified if compared to that of the arginine-deficient animals.

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SITES OF FORMATION OF PLASMA PHOSPHOPROTEIN AND PHOSPHOLIPID IN THE ESTROGENIZED COCKEREL[1]

W. E. VANSTONE, D. G. DALE, W. F. OLIVER, AND R. H. COMMON

Abstract

Estrogen increased plasma Ca, but not plasma total protein, phosphoprotein P, or phospholipid P, in functionally hepatectomized cockerels. Estrogen increased all four plasma constituents in sham-operated cockerels. Functional hepatectomy of the estrogenized cockerel abolished incorporation into plasma phosphoprotein of injected orthophosphate labelled with P^{32}, and reduced but did not abolish incorporation of P^{32} into plasma phospholipid. These observations are submitted as direct evidence that plasma phosphoprotein in the estrogenized cockerel is exclusively hepatic in origin.

Introduction

Miller and his associates (7, 8, 9) have presented direct evidence that the liver is the site of synthesis of all plasma albumin and fibrinogen and of most, if not all, of the α- and β-globulins in the rat. It has been demonstrated also that practically all plasma phospholipid in the unfed dog originates in the liver (2, 4). The chicken liver is capable of serum albumin production *in vitro* (11, 12) and has been shown to be a major source of plasma phospholipid *in vivo* (13, 15).

The onset of reproductive activity in pullets leads to a striking increase of the levels of serum calcium and plasma phospholipid and to the appearance *de novo* of plasma phosphoprotein (serum vitellin). The treatment of cockerels or sexually immature pullets with estrogen leads to similar effects. The increase in serum calcium occurs mainly in the non-ultrafiltrable fraction, and most investigators, e.g., McDonald and Riddle (6), have related the increase to increments in the levels of both colloidal calcium phosphate and vitellin-bound calcium. However, Schjeide and Urist (18) have suggested recently that by far the greater part of the increment of serum calcium in the sera of heavily estrogenized roosters is associated with phosphoprotein. These investigators showed that practically all of the serum calcium in such birds could be accounted for as calcium complexed with phosphoprotein, together with smaller amounts complexed with albumin and lipoprotein. Schjeide and Urist pointed out that, if their observations are correct, relatively little calcium would be available for the formation of colloidal calcium phosphate.

Roepke and Bushnell (17) and Hosoda *et al.* (5) have produced serological evidence that serum vitellin is produced by somatic tissues in the laying hen and not by the gonad. It has, of course, been known for a long time that the ovary is not needed for serum vitellin production, because estrogen

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evokes production of serum vitellin in males and in castrates of either sex. More recently, Flickinger and Rounds (3) have presented evidence based on the use of P^{32} which indicated that phosphoprotein is synthesized by the maternal organism and is transported to the ovarian eggs by the blood stream. Hosoda *et al.* (5) and Flickinger and Rounds (3) concluded that serum vitellin is probably synthesized in the liver.

Ranney *et al.* (14) perfected the technique of functional hepatectomy of the domestic fowl. In this procedure, exteriorized ligatures are placed around the afferent hepatic vessels, and the birds are allowed to recover from the operation for a few days. Functional hepatectomy is performed by tightening the ligatures. We have found it desirable to sew the ends of the ligatures under the skin, otherwise the birds are liable to hepatectomize themselves in the course of preening. Ranney *et al.* subsequently used their technique to demonstrate, firstly, that the liver is the source of approximately 61% of the plasma phospholipid in the non-estrogenized cockerel (15) and, secondly, that the liver is of major importance in the augmentation of plasma phospholipid in the estrogenized cockerel (13).

Hepatectomy experiments similar to those of Ranney *et al.* (13, 15) have been undertaken with three purposes in mind: firstly, to demonstrate by a direct experimental technique that plasma phosphoprotein originates in the liver; secondly, to determine the effect of functional hepatectomy on estrogen-induced hypercalcemia; and thirdly, to determine the importance of the liver, relative to extrahepatic tissues, in the synthesis of plasma phosphoprotein and phospholipid in estrogenized cockerels. Two experiments are described below.

Experimental

Experimental Materials

Crossbred cockerels (New Hampshire ♂ × Barred Plymouth Rock ♀), between 12 and 16 weeks of age, were placed in individual cages for 2 days before the beginning of each experiment. Each bird was offered water and a developing ration ad libitum throughout the experiment.

Estradiol benzoate ('Progynon B,' Schering) was donated by the Schering Corporation Limited, Montreal.

Disodium phosphate (labelled with P^{32} in the orthophosphate form) was obtained from the Atomic Energy Authority, Harwell, England, through Atomic Energy of Canada, Limited.

General Technique and Analytical Methods

Functional hepatectomies were performed by a two-stage operative technique (14). Blood samples were collected in small flasks containing sodium heparin (10 ml. blood into 200 U.S.P. units (0.2 ml. aqueous solution) of heparin). Hematocrit determinations were made on samples of well-mixed heparinized blood. The balance of the heparinized blood was subjected to mild centrifugation and the plasma was decanted.

Total plasma protein was determined by the biuret reaction (22). Calcium was determined by a combination of the methods of Halverson and Bergeim and of Stanford and Wheatley (10).

Phospholipid and phosphoprotein fractions were obtained in experiment No. 1 by Schneider's method (19). The following modification of Schneider's method was employed in experiment No. 2 to obtain these fractions: Suitable portions of plasma were repeatedly extracted with 5% (w/v) trichloroacetic acid, water, boiling chloroform–methanol (1 : 1 v/v), and hot 5% (w/v) trichloroacetic acid. The chloroform–methanol extracts were combined and the lipid fraction was purified as described by Dawson (1). The phosphorus of the final protein residue was designated phosphoprotein P.

The method of Shelton and Harper (20) was employed for the digestion and determination of the phosphorus content of the samples, using sulphuric acid rather than perchloric as one of the possibilities suggested by the authors. Sulphuric acid was added to the digested samples and heating was continued to drive off nitric and perchloric acids. The digests were made just alkaline to *p*-nitrophenol with ammonia and then just acid to the indicator with 1.0 *N* H_2SO_4. The samples were then cooled and made to volume with distilled water. Aliquots for color development were placed in large test tubes graduated at 10 ml. The volume of the aliquot was made to approximately 7.5 ml. with distilled water, 0.2 ml. molybdate solution (20 g. $Na_2MoO_4.2H_2O$ + 200 ml. concentrated H_2SO_4 made to 1 liter with distilled water) was added, and the solution well mixed, and 0.2 ml. hydrazine solution (20 g. $NH_2NH_2.H_2SO_4$ made to 1 liter with distilled water) was then added. The solution was again mixed and the tubes were placed in a boiling water bath for 5 minutes. The tubes were then cooled and the contents made to 10 ml. with distilled water. Percentage transmission of the samples was measured in an Evelyn photoelectric colorimeter (660 mμ filter).

Portions, each consisting of 2 ml. of the digested, neutralized samples, were placed in steel counting cups and counted immediately under an end window Geiger–Mueller tube.

Experiment No. 1

Six cockerels were weighed and prepared for hepatectomy on July 13 and one bird (No. 91) was prepared on July 15. At 2130 hours on July 16 each bird was weighed and then received, by intramuscular injection, 10 mg. estradiol benzoate in 3 ml. sesame oil (0.5 ml. into each of two spots on each breast and 0.5 ml. into each thigh). At 1330 hours on July 17 each bird received 3.33 mg. estradiol benzoate in 1 ml. sesame oil (0.5 ml. into each breast). Blood samples, 10 ml., were obtained by puncture of the wing veins at 1530 hours. Immediately after the samples were taken, the ligatures were tightened in five of the birds, the remaining two birds serving as sham-operated controls in which the ligatures were disturbed but not tightened. At 1730 and 1930 hours each bird received 1 ml. of a 20% (w/v) glucose solution by intraperitoneal injection. At 2130 hours the birds were bled from the jugular veins and decapitated.

The results are presented in Table I. All birds, except No. 91, were heavier just before the second operation than they were just before the first operation. The hematocrit values for all birds were lower at the time of drawing the second blood samples than they had been six hours earlier. This was to be expected, since Sturkie and Newman (21) found that the act of taking a blood sample from fowl, even with samples as small as 2.0 ml., resulted in hemodilution. The hemodilution which occurred was of the same order of magnitude in both groups.

When the results are corrected for hemodilution on the assumption that total cell volume was not affected by blood sampling or operative procedure, it is seen that total plasma protein, calcium, phosphoprotein P, and phospholipid P continued to increase in the sham-operated birds during the experimental period. In the hepatectomized birds, plasma calcium also continued to increase, though possibly at a lesser rate, while total plasma protein remained relatively steady. During the same period, phosphoprotein P and phospholipid P decreased in the hepatectomized cockerels. This decrease was more pronounced in the case of phosphoprotein P; this may be a reflection of the relatively greater rapidity of turnover of phosphoprotein, but it may also reflect a greater degree of dependence of phosphoprotein synthesis on liver function.

TABLE I

EFFECT OF FUNCTIONAL HEPATECTOMY ON THE ESTROGEN-INDUCED CHANGES OF CERTAIN BLOOD CONSTITUENTS IN THE COCKEREL*

	Post-ligation time, hr.	Sham-operated, Bird No.		Hepatectomized, Bird No.†				
		82	87	84	85	86	88	91
Live weight, kg.,								
1st operation		1.33	1.30	1.31	1.33	1.21	1.15	1.64
2nd operation		1.42	1.33	1.43	1.46	1.32	1.26	1.64
Hematocrit	0	29	31	28	30	31	27	33
	6	21	25	25	22	21	20	30
Plasma protein, g./100 ml.	0	3.7	3.7	3.6	3.8	3.5	3.9	3.5
	6	3.1	3.3	3.0	2.9	2.6	2.8	3.4
		(4.3)	(4.1)	(3.4)	(4.0)	(3.8)	(3.8)	(3.7)
Plasma calcium, mg./100 ml.	0	12.9	10.4	15.6	11.7	11.8	11.6	11.9
	6	18.1	14.7	16.9	12.4	13.6	11.6	12.6
		(25.0)	(18.2)	(18.9)	(16.9)	(20.1)	(15.7)	(13.9)
Plasma protein P, mg./100 ml.	0	0.9	1.0	1.0	0.7	0.9	0.9	0.8
	6	0.8	1.0	0.4	0.2	0.5	0.2	0.4
		(1.1)	(1.2)	(0.4)	(0.3)	(0.7)	(0.3)	(0.4)
Plasma lipid P, mg./100 ml.	0	12.5	10.9	11.2	12.0	9.8	10.9	8.0
	6	12.0	10.3	8.8	8.4	6.3	7.4	6.8
		(16.6)	(12.8)	(9.8)	(11.5)	(9.3)	(10.0)	(7.5)

*Figures in parentheses are the 6-hour values corrected for hemodilution.

†Bird No. 91 was allowed to recover for only 1 day before the first injection of estrogen. The remainder of the birds were allowed to recover for 3 days.

Experiment No. 2

Ten cockerels were weighed and were then prepared for hepatectomy. At 2100 hours on each of the following 3 days each bird received, by intramuscular injection, 6.66 mg. estradiol benzoate in 2 ml. sesame oil. At

0900 hours on the fourth day each bird was weighed and then given 3.33 mg. estradiol benzoate in 1 ml. sesame oil by the same route. One hour later the ligatures of one half of the birds were tightened. The remainder of the birds served as sham-operated controls in which the ligatures were disturbed but not tightened. Immediately thereafter each bird received approximately 149μc. P^{32} (0.88 mg. Na_2HPO_4 in 0.5 ml. aqueous solution) by intravenous injection. Two and four hours after ligation each bird was given 1 ml. of a 20% (w/v) solution of glucose by intraperitoneal injection. Six hours after ligation each bird was bled from the jugular vein and was then decapitated.

The results of experiment No. 2 are presented in Table II. The plasma phosphoprotein of the sham-operated cockerels had an average specific activity of 25.4 cts./min./μg. phosphorus as against a figure of 0.1 for the hepatectomized birds. The authors believe that the slight activity found in the latter birds arose from contamination, and that the activity was substantially nil. Accordingly, these observations may be considered as strong support for the view that synthesis of plasma phosphoprotein in the estrogenized bird is exclusively hepatic.

TABLE II

EFFECT OF FUNCTIONAL HEPATECTOMY ON THE INCORPORATION OF RADIOACTIVE INORGANIC PHOSPHORUS INTO PLASMA PHOSPHOPROTEIN AND PHOSPHOLIPIDS OF THE ESTROGENIZED COCKEREL

Bird No.	Live weight, kg.		Specific activity*			
			Plasma phosphoprotein		Plasma phospholipid	
	At 1st operation	At 2nd operation	Duplicate	Mean	Duplicate	Mean
			Sham-operated			
92	1.50	1.60	29.1 24.0	26.6	5.1 5.3	5.2
93	1.44	1.58	26.1 28.3	27.2	4.3 4.0	4.2
94	1.20	1.30	29.5 24.7	27.1	7.5 7.8	7.6
95	1.25	1.35	23.2 24.9	24.0	5.5 5.8	5.6
97	1.60	1.60	21.2 23.2	22.2	5.4 5.5	5.4
			Hepatectomized			
89	1.61	1.64	0.0 0.4†	0.2	1.1 1.7	1.4
90	1.21	1.37	0.0 0.0	0.0	1.0 1.2	1.1
96	1.34	1.30	0.0 0.0	0.0	1.9 1.0	1.4
98	1.31	1.39	0.5† 0.0	0.2	1.2 1.2	1.2
99	1.67	1.79	0.0 0.0	0.0	1.1 1.3	1.2

*Specific activity is expressed as counts/min./μg. P.
†Believed to be due to contamination of the phosphoprotein residue.

The results for this experiment also show that plasma phospholipid became labelled to a greater extent in the sham-operated birds than in the hepatectomized birds. At the same time, and in confirmation of the results of Ranney *et al.* (13, 15) but in contrast to the results for plasma phosphoprotein, there was appreciable labelling of plasma phospholipid in the hepatectomized cockerel.

Discussion

These experiments leave little doubt that liver synthesis is the only source of serum vitellin in the estrogenized cockerel. It was rather surprising to find that functional hepatectomy, which completely inhibited the formation of vitellin, had only a slightly depressant effect on the estrogen-induced hypercalcemia.

Most investigators have predicated that the increased calcium is present in blood as both vitellin bound and colloidal calcium phosphate, while Schjeide and Urist (18) have reported that practically all of the serum calcium in estrogenized roosters may be complexed with vitellin. However, the present experiments have shown that, in the estrogenized hepatectomized cockerel, there was no correlation between the levels of plasma calcium and vitellin and may indicate that, in these birds at least, the increased calcium is entirely due to increased colloidal calcium phosphate.

Ranney *et al.* (13, 15) showed that the liver must be the major source of plasma phospholipid in both estrogenized and non-estrogenized cockerels. However, they found that functional hepatectomy of the non-estrogenized cockerel only reduced the specific activity of the plasma phospholipid to 39% of that found in sham-operated controls (15). In the results now reported for estrogenized cockerels, the specific activity of plasma phospholipid phosphorus was only 23% of that found in similarily injected sham-operated control birds. This is in general agreement with the results of Ranney *et al.* (15) and to that extent supports the theory that, although plasma phospholipid may originate elsewhere than in the liver, nevertheless the liver is the major site of origin. It is, perhaps, permissible to speculate on the circumstance that the specific activity of the plasma phospholipid P was decreased to a relatively greater degree by hepatectomy in the present work than in the experiment of Ranney *et al.* (15). This difference is compatible with the view that the liver is more deeply involved, relative to extrahepatic tissues, in the estrogen-induced phospholipemia than it is in the maintenance of normal phospholipid levels. In this connection it should be recalled that the estrogen-induced phospholipemia in the bird preferentially affects the levels of lecithin and cephalin, the level of sphingomyelin exhibiting little if any change (16).

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THE EFFECTS OF BARBITURATES ON PARTIALLY ISOLATED REGIONS OF THE CENTRAL NERVOUS SYSTEM[1]

J. J. Seguin and G. W. Stavraky

Abstract

In 52 cats in which the left frontal lobe (including the motor cortex) or the whole cerebral hemisphere had been removed 3 months to 2 years previously, sodium pentobarbital (i.v., 1.0–2.0 mg./kg.) caused weakness and in-co-ordination in the decentralized limbs. Larger quantities of pentobarbital (3.0–8.0 mg./kg.) made the animals unable to stand ('standing test') for 13.0 ± 1.8 minutes while 25 control cats exhibited this effect for 5.6 ± 1.5 minutes. Using the 'standing test' as a criterion the median effective dose of pentobarbital was found to be 1.7 mg./kg. for the operated group and 3.7 mg./kg. for the controls, the difference being statistically significant. Sodium phenobarbital had similar effects.

Injected (i.m.) 1 hour before pentylenetetrazol (10 mg./kg.) in 21 operated and 19 control cats, pentobarbital (1.0–6.0 mg./kg.) reduced the incidence and severity of convulsions in the operated cats to a greater extent than in the controls, as judged by a conversion of the clonic–tonic–clonic fits into purely clonic seizures.

In 23 spinal cats in which the spinal cord was semisected 9 to 39 days previously, the excitability of the motoneurones was tested by means of intra-aortal injections of acetylcholine (ACh, 0.03–3 mg./kg.). Small quantities of pentobarbital occasionally augmented the responses to ACh whereas larger quantities of pentobarbital (0.3–11.0 mg./kg.) or phenobarbital (0.7–6.0 mg./kg.) had a depressant effect, both actions being more marked on the semisected side. The significance of these findings is discussed.

Introduction

Incidental observations made on animals with various cerebral lesions indicated that such animals are more susceptible to depressant agents than intact ones (Morita (11); Mettler and Culler (10); Stavraky (13); Drake and Stavraky (5); Wikler (15, 16); Ross, Leavitt, Holst, and Clemente (12); Dasgupta, Mukherjee, and Werner (2); and Jones and Lombroso (7)). The present investigation was undertaken in order to test the effects of sodium pentobarbital and sodium phenobarbital on partially isolated neurones in the central nervous system, and to ascertain the susceptibility of animals with cerebral ablations of long standing to the depressant and anticonvulsant action of these drugs.

Methods

Fifty-two operated cats were used (31 frontal and 21 semidecerebrated) as well as 25 controls. A random selection of cats of both sexes was made from a group of full grown animals in all experiments. Left-sided ablations were done, the motor cortex always being included in the removal. The barbiturates were injected into the femoral vein through a 24-gauge needle, the duration and volume of the injection being kept constant throughout. The mean latency and duration of various effects were calculated with their standard

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errors; differences between groups and between responses of the two sides were tested for significance by means of Fisher's '*t*' test (6) and the median effective dose of sodium pentobarbital was calculated for the operated and control animals by the method of Litchfield and Wilcoxon (8).

In another set of experiments aseptic semisection of the spinal cord was carried out at the L_1 level in 23 cats. Nine to 39 days later the brain and medulla were pithed under ether anesthesia, artificial respiration was induced, the sciatic and obturator nerves were cut bilaterally, and following appropriate fixation of the femurs the contractions of both quadriceps muscles were recorded by means of isotonic myographs. After complete recovery of the animals from anesthesia, the tested drugs were introduced through a polyethylene catheter inserted by way of the subclavian artery into the appropriate level of the aorta.

Acetylcholine bromide – ACh (Eastman-Kodak), sodium pentobarbital (Nembutal, sodium powder, Abbott), sodium phenobarbital (Merck), pentylenetetrazol (Metrazol, Bilhüber-Knoll Corp.), and strychnine sulphate (Parke-Davis) were used throughout.

Results

I. The Effects of Sodium Pentobarbital and Sodium Phenobarbital on Frontal-Lobectomized or Semidecerebrated Cats

A. Sodium Pentobarbital

Sodium pentobarbital was injected intravenously, in doses ranging from 1.2 to 8.0 mg./kg. Each dose of pentobarbital was given only once to each animal and, when two different doses were given to the same cat, there was an interval of at least 2 weeks between the injections. In the control animals injections of 2.0 to 8.0 mg./kg. of pentobarbital resulted in an unsteady gait, the animals falling and staggering indiscriminately to either side, and in the case of higher doses they were often unable to stand or walk for variable periods of time ranging from 2 to 46 minutes.

In spite of considerable differences in the susceptibility of individual animals, the injection of even the smallest tested quantity (1.2 mg./kg.) of pentobarbital caused the operated cats to lean, stagger, or fall, invariably away from the side of the operation. The animals dragged their right forepaws when walking, and stepped on their dorsa while the right hind paws slid out from under them. When larger quantities of sodium pentobarbital (2.0 to 8.0 mg./kg.) were injected, a greater weakness and in-co-ordination developed on the right side, and the animals were unable to stand unassisted for from 2 to 120 minutes. When the cat was finally able to stand and walk it still showed signs of weakness and in-co-ordination on the right side and often fell to the right during recovery (Figs. 1 and 2). The inability of a limb to support the weight of the animal was taken as a criterion of depression. All four limbs were tested individually in rotation by supporting the animal manually in the upright position and then lowering it gradually so that its weight rested on the limb which was tested. The duration of depression was timed to the nearest

minute when the limb could once again support the body weight without collapsing. The depression times thus determined were the same on both sides in the control group and were shorter than those on either side in the operated group (Table I). Furthermore, in the operated cats the right extremities were depressed for much longer periods of time than the left. These differences were statistically significant.

FIG. 1. Effect of 5 mg./kg. of sodium pentobarbital (i.v.) in a cat 22 months after the removal of the left frontal lobe including the motor cortex.

Before the injection, animal walked normally and when raised by the hind limbs supported its weight equally on both forepaws. The only effect of the operation was the absence of 'placing reactions' in the right extremities (A). After the injection the cat was unable to walk for 12 minutes. When, during this time, the pelvis was raised, the cat supported its weight only on the left forepaw, the right remaining limp (B). During the following 25 minutes, the animal walked leaning, staggering, and falling to the right, marked weakness and in-co-ordination of the right limbs being in evidence.

FIG. 2. Effect of 5 mg./kg. of sodium pentobarbital (i.v.) in a cat 21 months after the removal of the complete left cerebral hemisphere.

Before the injection the animal walked almost normally but circled to the left when excited. Eight seconds after the injection the cat fell to the right and was unable to stand or walk for 20 minutes, showing signs of weakness and in-co-ordination in the right limbs for 30 minutes. When supported from the right side it could stand on the left extremities (A); when supported from the left side the right limbs collapsed under the weight of the body (B).

The mean values for the duration of the different effects of pentobarbital are presented in Table II. From the figures collected in this table it was established that in both groups of cats there was a significant ($P < 0.05$) decrease in latency when the quantity of sodium pentobarbital was increased from 2.0 to 5.0 mg./kg. The mean duration of the inability to stand ('standing test') was also increased. In the operated cats this latter effect was much

more pronounced than in the controls, and the mean time of the 'standing test' was significantly greater in the operated group with the 5.0 and 8.0 mg./kg. doses of pentobarbital than with the 2.0 mg./kg. ($P < 0.02$ and $P < 0.001$ respectively). While the same trend was present in the control cats, it was not sufficiently pronounced to be statistically valid. When the 'standing test' time was compared in the two groups, it was found to be consistently longer for the operated animals than for the controls, the difference being significant at the 5 mg./kg. level. When the number of cats in the control and operated groups was equalized at each dose level using a random selection and when the 'standing test' times for the 2.0, 3.2, and 5.0 mg./kg. doses were combined, it was found that the operated cats were unable to stand for 13.0 ± 1.8 minutes as compared to 5.6 ± 1.5 for the controls, the P value for the difference being less than 0.01.

TABLE I

DURATION OF DEPRESSION IN THE LIMBS OF CONTROL AND OPERATED CATS INDUCED BY SODIUM PENTOBARBITAL*

	Mean depression time (minutes ±S.E.M.)			
	Front limbs		Hind limbs	
	Right	Left	Right	Left
Operated cats	6.7±1.07	3.9±1.01	5.3±0.85	4.2±1.15
	↑ (P <0.01)†		↑ (P <0.05)†	
	(P >0.01)		(P >0.05)	
Control cats	↓ 2.4±0.65		↓ 2.9±0.68	

*Combined results of injections of 2.0, 3.2, and 5.0 mg./kg. of sodium pentobarbital, into nine control and nine operated cats (number of cats at each dose level equalized).

P values: †Comparison of mean depression time in right limb with that of left limb in operated cats.

Vertical arrows—comparison of mean depression time in right limbs of operated cats with that of control cats.

The period when the cats showed signs of weakness or in-co-ordination ('total period of depression') also became longer when the doses of sodium pentobarbital were increased. Thus, when the effect of the 2.0 mg./kg. dose was compared with that of 5.0 and 8.0 mg./kg. the difference was highly significant, the P values ranging from less than 0.001 to 0.05 for both the operated and control group. As with the 'standing test' this period lasted longer in the operated cats than in the controls, but owing to wide variations among individual animals the difference between the two groups was not statistically significant.

In view of the indications of an apparently greater susceptibility of cats with cerebral ablations to the depressant action of pentobarbital the median effective doses (ED_{50}'s) of this drug were determined for control cats and for cats in which the left frontal lobe was removed 24 to 36 months previously. The 'standing test', defined previously as the ability or inability of the animals

TABLE II

MEAN VALUES (±S.E.M.) FOR LATENT PERIOD AND FOR THE DURATION OF DEPRESSANT EFFECTS PRODUCED BY SODIUM PENTOBARBITAL IN CONTROL (C) AND OPERATED (O) CATS

Sodium pentobarbital (mg./kg. i.v.)	Latent period (seconds)				Standing test* (minutes)				Total period of depression† (minutes)			
	C		O		C		O		C		O	
	No.	Duration	No.	Duration	No.	Duration	No.	Duration	No.	Duration	No.	Duration
1.2			4	10.3±3.35			2	4.0±1.0			4	12.0± 6.9
2.0	7	14.1±2.24	16	10.5±0.98	1	2.0	6	5.3±2.5	7	5.7±3.3	20	16.1± 2.4
3.2	6	10.3±1.69	12	11.1±1.90	3	4.2± 0.4	16	6.2±1.2	6	21.7± 7.4	22	17.7± 2.0
5.0	7	7.6±0.84	23	7.8±0.32	5	6.2± 2.3	16	13.6±1.5	7	27.7± 7.1	23	36.7± 5.0
8.0	2	11.0±0.0	11	7.1±0.77	2	28.5±17.5	10	47.6±9.5	2	110.0±10.0	11	130.0±11.0

*'Standing test' = stage of depression during which the cat was unable to stand or walk.
†'Total period of depression' = includes staggering, falling, and leaning as well as inability to stand or walk.

to stand following the intravenous injection of pentobarbital, was taken as a criterion of the effectiveness of this drug. This test was chosen because it offered the most definite end point of any of the effects of pentobarbital which was common to both the operated and control cats. The ED_{50} and the potency ratio were calculated in an experiment carried out on 21 control and 21 operated cats. They were divided into three groups of seven each and after a preliminary determination of the effective dose range was carried out on other groups of animals, pentobarbital was injected in logarithmically increasing doses. The most suitable quantities for the operated animals were found to be 1.2, 2.0, and 3.2 mg./kg., causing inability to stand in 29, 57, and 86% of the animals respectively; and for the control cats 2.0, 3.2_T and 5.0 mg./kg., which resulted in this effect in 14, 43, and 71% of the animals. The ED_{50} of pentobarbital for the operated cats was 1.7 mg./kg. (95% confidence limits of 1.1 to 2.5 mg./kg.) and for the controls it was 3.7 mg./kg. (95% confidence limits of 2.5 to 5.6 mg./kg.). A comparison of these doses shows that pentobarbital had a significantly greater depressant action on the operated animals, depressing them 2.2 times as much as the controls.

B. Sodium Phenobarbital

The effects of intravenous injections of sodium phenobarbital (5–50 mg./kg.) on 23 frontal-lobectomized and semidecerebrated cats were similar to those of sodium pentobarbital. Doses of phenobarbital larger than those of pentobarbital were needed to produce a comparable degree of in-co-ordination and the effects came on more gradually, lasted longer, and were less clearly defined. Doses of 5 to 19 mg./kg. produced mild in-co-ordination in 8 out of 10 animals which leaned, staggered away from the side of the operation, and dragged the paws on that side for from 6 minutes to 1 hour. An injection of 20 to 29 mg./kg. of phenobarbital resulted in marked weakness of the extremities contralaterally to the ablation in six cats. Four animals in this group staggered and fell repeatedly away from the side of the operation for 1 to 2 hours, while the remaining cats fell and were unable to get up for the same period of time. Seven animals which were injected with 30 to 50 mg./kg. of phenobarbital all fell away from the side of the operation and were unable to stand or walk for 1 to 8 hours. In two animals which were given 50 mg./kg. each, some in-co-ordination of the corresponding extremities persisted for 2 days.

C. Depression by Sodium Pentobarbital of Pentylenetetrazol-Induced Convulsions

In a separate set of experiments the effects of pentobarbital on pentylenetetrazol-induced convulsions were studied in 21 operated cats (13 left frontal-lobectomies and eight semidecerebrations of 4 months to 2 years standing) and in 19 controls. Pentylenetetrazol (10 mg./kg.) was injected intravenously once a week for four injections in combination with increasing quantities (1, 2, 4, and 6 mg./kg.) of sodium pentobarbital given intramuscularly 1 hour before the pentylenetetrazol. A 10 mg./kg. dose of pentylenetetrazol was

decided upon because it had previously been found to produce convulsions in 97% of a large group of operated animals and in 87% of the controls (Drake, Seguin, and Stavraky (4)). When given alone 2 weeks before the beginning of the experiment, this dose of pentylenetetrazol convulsed all the cats chosen for the experiment; the duration and distributions of the convulsive patterns were, however, the same as described by Drake, Seguin, and Stavraky (4, pp. 691–699) in the original large group of animals.

Under the influence of pentobarbital in the controls, a dissociation of the convulsive patterns often took place. The hind limbs developed a clonus while the front limbs went through a tonic phase.

In 13 operated cats the initial tonus of the right limbs was absent. In seven of them a symmetrical clonus marked the beginning of the convulsion, but in six cats a unilateral clonus on the left side was observed with a complete suppression or shortening of the convulsions on the right side of the body. This indicated a reversal of the usual trend. In two animals the rolling also showed a tendency toward reversal; the cat began to roll as usual to the right side (3) but later in the convulsion changed the direction of rolling to the left.

The effect of pentobarbital on the convulsability of the animals is presented in Table III. With an increase in the quantities of pentobarbital, the number of convulsions induced by pentylenetetrazol decreased in both the operated and control groups. However, at 1 and 2 mg./kg. doses the total number of convulsing animals was greater in the operated group; with 4 and 6 mg./kg. of pentobarbital this trend was reversed, and more control than operated cats convulsed. Particularly striking was the decrease in the number of clonic–tonic–clonic convulsions in the operated cats under the influence of pentobarbital. When more than 2 mg./kg. of this drug was given, all the convulsions in the operated group became clonic, while the same result in the control group was achieved only at the 6 mg./kg. dose level.

TABLE III

SENSITIVITY OF CONTROL (C) AND OPERATED (O) CATS TO PENTYLENETETRAZOL (10 MG./KG. I.V.) FOLLOWING INCREASING QUANTITIES OF SODIUM PENTOBARBITAL (1 INJECTION/DOSE/ANIMAL)

Sodium pentobarbital (mg./kg. i.m.)	Number of cats injected		% of C.T.C.* convulsions		% of Cl.* convulsions		C.T.C. and Cl. convulsions	
	C	O	C	O	C	O	C	O
0	19	21	58	76	42	24	100	100
1	17	20	47	30	18	40	65	70
2	14	20	7	25	43	35	50	60
4	12	21	16.7	0	16.7	14	33	14
6	9	21	0	0	11	5	11	5

*C.T.C. = clonic–tonic–clonic convulsions.
Cl. = clonic convulsion.

The latent period between the injections of pentylenetetrazol and the convulsions was not significantly altered in the controls (Table IV). On the other hand, in the operated cats, it became progressively longer with increasing doses of pentobarbital, the P value for the difference between the 0 dose and 2 and 4 mg./kg. being less than 0.05. Similarly, the duration of the convulsions was markedly lengthened in the operated cats when pentylenetetrazol was preceded by pentobarbital; in the control group this prolongation of the seizures was only slight and was not statistically significant. The increase in the duration of the convulsions which took place under the influence of 1 mg./kg. of pentobarbital was apparently due to the fact that a large number of convulsions were changed from a relatively short but intense clonic–tonic–clonic type of convulsion to a more prolonged but less severe clonic seizure.

TABLE IV

EFFECT OF SODIUM PENTOBARBITAL ON MEAN LATENCY AND DURATION (SECONDS ± S.E.M.) OF PENTYLENETETRAZOL (10 MG./KG. I.V.) INDUCED CONVULSIONS

Sodium pentobarbital (mg./kg. i.m.)	Mean latency		Mean duration		Number of convulsions (C.T.C. and Cl.)	
	C	O	C	O	C	O
0	6.8±0.28	5.6±0.29 (P <0.01)†	37.3±3.15	42.2±1.42	19	18
1.0	6.6±0.32	6.2±0.27	40.1±2.62	79.7±9.52** (P< 0.001)†	11	12
2.0	6.0±0.32	6.6±0.37*	41.6±4.34	65.4±5.42** (P <0.01)†	7	10
4.0	7.0±1.0	7.3±0.36*	42.5±7.5	52.3±5.36	2	3
6.0			40	75	1	1

*Differs significantly from 0 dose level (P <0.05).
**Differs significantly from 0 dose level (P <0.001).
†Significance of difference between control and operated groups.

II. ANALYSIS OF THE EFFECTS OF SODIUM PENTOBARBITAL AND SODIUM PHENOBARBITAL IN SPINAL CATS WITH PRECEDING SEMISECTION OF THE CORD

The effects of pentobarbital were tested on the background of the responses induced by ACh in 18 cats and reversals were observed in 15 of them. This method of testing the sensitivity of spinal motoneurones by ACh was devised by Cannon and Haimovichi (1) while Drake, Seguin, and Stavraky (4) showed that intra-aortal injections of this agent in quantities used in the test do not affect the systemic blood pressure to any significant degree and that the effects of ACh should be attributed to its direct action on the spinal cord. As shown in Fig. 3A, before the administration of barbiturates the threshold dose of ACh was less for the previously semisected side and the contractions

of the quadriceps were more pronounced on that side when they occurred bilaterally (Fig. 4A). Discriminative doses of pentobarbital (0.3–11.0 mg./kg.) selectively depressed the chronically decentralized spinal neurones. This resulted in a reversal of the effects of ACh on the two sides (Figs. 3B and 4B). This effect of pentobarbital was transient; as seen in Fig. 4C, when ACh was injected 6 minutes after the pentobarbital it was again more effective on the sensitized side. Repeated injections of ACh or small quantities of strychnine seemed to hasten the return of the sensitivity on the semisected side.

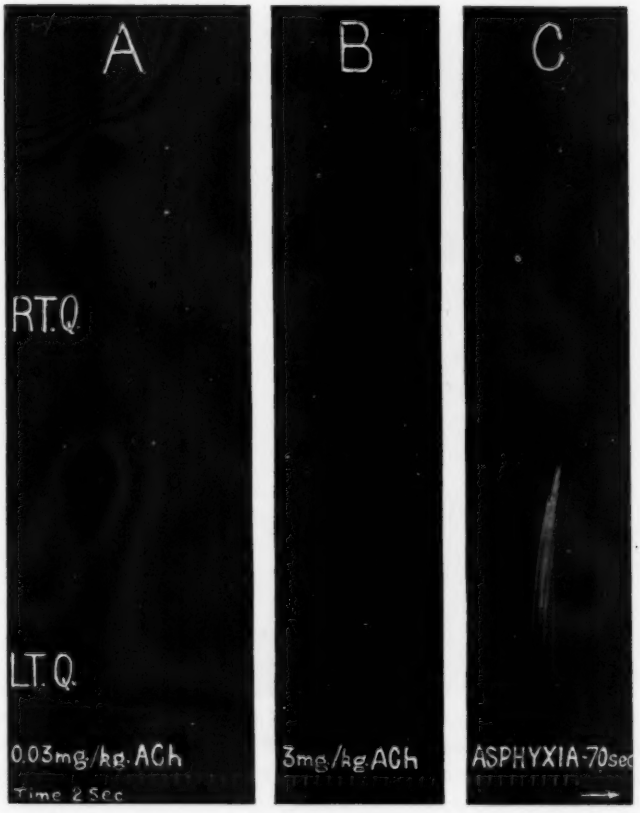

FIG. 3. Effects of sodium pentobarbital on responses of the quadriceps muscles to acetylcholine and asphyxia, in a high spinal cat 37 days after right semisection of the cord at L_1 level (Rt. Q., right quadriceps; Lt. Q., left quadriceps). All injections intra-aortal.

A—before pentobarbital, response limited to right quadriceps.

B—30 seconds after the injection of 6 mg./kg. of pentobarbital reversal of the response to ACh.

C—2 minutes after the same injection of pentobarbital asphyxia gave a similar result (before pentobarbital asphyxia was more effective on the right side—see text).

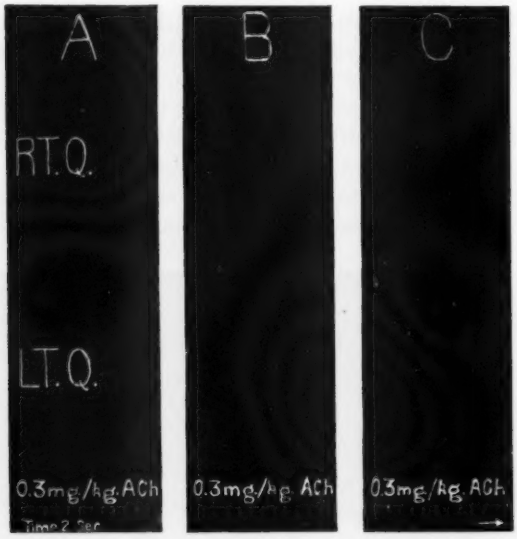

FIG. 4. Effect of clamping the abdominal aorta (same experiment as in FIG. 3; all injections intra-aortal).
A—control (aorta open).
B—2 minutes after 0.6 mg./kg. of pentobarbital (injections with aorta clamped at the bifurcation of the iliac arteries).
C—control 6 minutes later (aorta open).

The possibility of a direct effect of acetylcholine or of pentobarbital on the muscles was ruled out in experiments in which a temporary clamping off of the abdominal aorta was carried out at the bifurcation of the iliac arteries (Fig. 4). Under these conditions the drugs were prevented from reaching the quadriceps muscles but the results of the injections were the same as before.

Cannon and Haimovichi (1) showed that asphyxia was also more effective in producing muscular contractions on the side of the preceding semisection of the spinal cord. This was confirmed in the present study, and it was found that pentobarbital depressed the isolated neurones to the extent that asphyxia induced contractions of the quadriceps occurred only on the control side (Fig. 3C).

While appropriate doses of pentobarbital had a depressing effect on spinal neurones, very small quantities of this drug (0.3–0.7 mg./kg.) exerted an excitatory action. This effect was very pronounced on the semisected side in experiments in which ACh was used as a stimulating agent. When the dose of pentobarbital was more effective it caused an increased excitability of the control side while exerting a depressing effect on the semisected side (Fig. 5).

The effects of sodium phenobarbital (0.7–6.0 mg./kg.) were studied in five cats and were similar to those of sodium pentobarbital. A typical reversal of the response to ACh brought about by phenobarbital and the subsequent restoration of the original relationship is shown in Fig. 6.

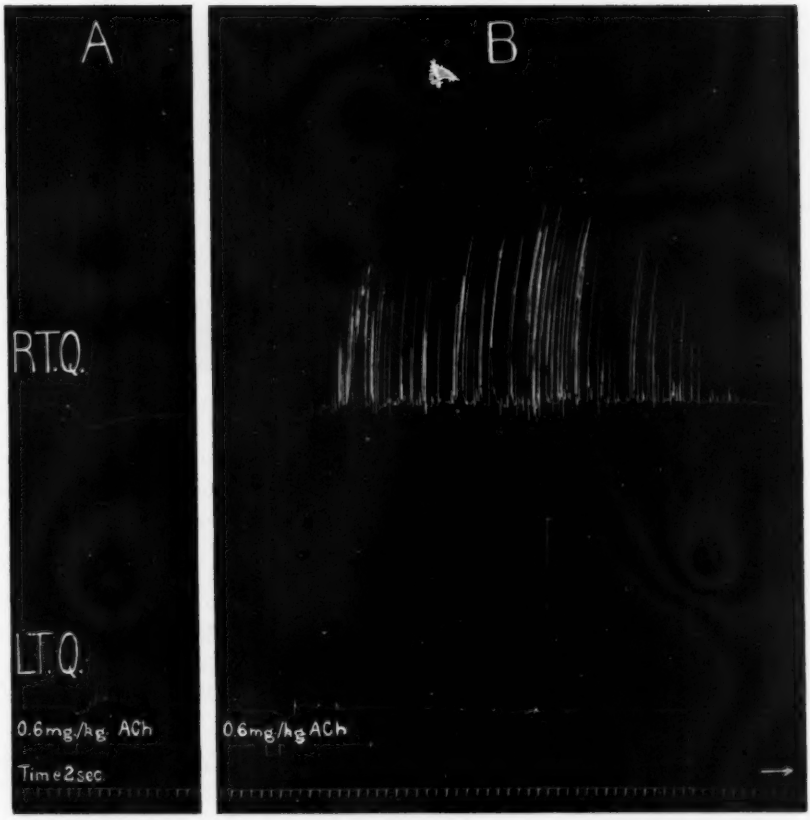

FIG. 5. Reversal of effects of ACh by sodium pentobarbital in a spinal cat 15 days after left semisection of the cord (all injections intra-aortal).

A—control.

B—22 sec. after 0.6 mg./kg. of sodium pentobarbital.

(Note depression of the response on semisected side and exaggeration on the control side.)

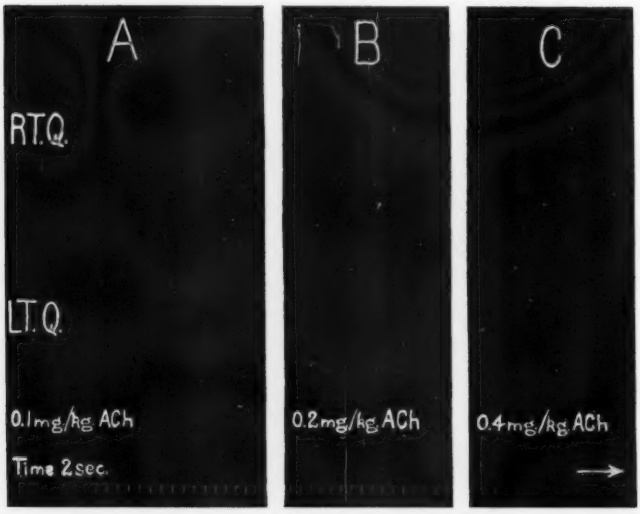

FIG. 6. Effects of sodium phenobarbital on responses of the quadriceps muscles to ACh in a high spinal cat 39 days after left semisection of the cord at L_1 level (all injections intra-aortal).

A—control.

B—12 minutes after 1.5 mg./kg. of phenobarbital.

(Note reversal of the effects of ACh on the two sides.)

C—restoration of initial relationship after several consecutive injections of ACh repeated in rapid succession.

Discussion

The results show conclusively that partial isolation of various regions of the central nervous system sensitizes these regions to barbiturates and leads to a greater effectiveness of these drugs in preventing or reducing the severity of generalized convulsions induced by chemical means.

In agreement with these findings, Dmitriev (3) observed in reptiles and amphibia that after a transverse section of the spinal cord, motor co-ordination gradually returned in the paralyzed limbs. This re-established activity was selectively depressed by chloral hydrate and urethane.

In the present study, in which unilateral lesions were made, it was possible to compare the depressant action of barbiturates in groups of normal and operated cats, showing a statistically significant difference in the responses of the two groups, and, by the use of the intact side of the animals as a control, to test the effectiveness of these drugs on the partially denervated side.

The application of the second technique indicated that very small quantities of barbiturates selectively augmented the sensitivity of isolated neurones while larger amounts of these drugs had a depressing effect. These results bring out the fact pointed out by McLennan and Elliott (9) that narcotics in small quantities can cause hyperactivity as well as a depression.

A lowered threshold for stimulating agents or an augmented excitability to nerve impulses reaching the partially denervated neurones by way of the remaining connections may be regarded as the result of 'disinhibition' or that of an 'augmented facilitatory influx'. Sensitization is usually distinguished from these possibilities by the gradual onset of the hyperexcitable state which takes place after severance of given anatomical connections. The fact that otherwise hyperexcitable neurones are more readily depressed by barbiturates than the fully innervated ones may be taken as additional proof that partial denervation renders nerve cells truly supersensitive to changes in their environment regardless of whether these changes lead to an excitation or to a depression.

The greater effectiveness of barbiturates on neurones whose excitability is enhanced may be of considerable practical importance. It was assumed for some time that anticonvulsant drugs in doses which have little effect upon normal neurones may have quantitatively important effects upon those which are pathologically hyperactive (Toman and Goodman (14)). An actual demonstration of this principle is provided in the presented experiments carried out on neurones sensitized by partial isolation. Toman and Goodman also suggested "that increased activity of normal brain tissues may inhibit discharges from seizure foci". An analogous situation is demonstrated at the present time in the fact that doses of pentobarbital which have a slight excitatory effect on intact neurones are sufficient to depress the supersensitive areas thus establishing a preferential balance of activity in the normal areas of the nervous system.

The relationship between sensitization by denervation and epileptogenous foci is by no means established, but the facts referred to above offer a method of study of the action of barbiturates on the interrelation of activity in neuronal pools of different sensitivity, a situation which is known to exist in clinical epilepsy.

Conclusion

From the presented results the conclusion can be drawn that partially isolated regions of the central nervous system acquire a greater than normal sensitivity to depressing agents. This finding may be of practical significance in the appreciation of the mode of action of sedative and anticonvulsant drugs. Also it emphasizes the fact that supersensitivity of partially isolated neurones cannot be regarded only as a hyperexcitable condition caused by a 'disinhibition' or by an 'augmented influx' but is a state of the nerve cells in which the latter become more sensitive to depressant as well as to excitatory influences.

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THE REASON FOR THE SHAPE OF THE DISTENSIBILITY CURVES OF ARTERIES[1]

MARGOT R. ROACH AND ALAN C. BURTON

Abstract

It is characteristic of arteries that they do not obey Hooke's law, but resist further stretch more strongly, the more they are stretched. It appears that this might be due to the combination of elastin fibers in the elastic laminae, with the much less distensible collagenous fibers in the media and adventitia, more and more of which reach their 'unstretched length' as distension is increased. This has been verified on human iliac arteries, from autopsy, by comparing the 'elastic diagrams' (tension vs. circumference) before and after differential digestion of collagen by formic acid, and digestion of elastin by crude trypsin (containing an elastase). This proved that the resistance to stretch at low pressures was almost entirely due to elastin fibers, that at physiological pressures due to both collagenous and elastin fibers, but dominantly to collagen, and that at high pressures almost entirely due to collagenous fibers. In future work on the effect of age on the elasticity of iliac arteries, the initial slope of the elastic diagram can be taken as an index of the state, or number, of the elastin fibers, and the final slope as an index of the state, or number, of collagenous fibers.

I. Introduction

'Elasticity' is that property of materials by which they develop a force, or 'tension', to resist deformation, and a 'modulus' of elasticity is the ratio of the elastic tension developed to the amount of deformation that elicited it. When the deformation is in one axis only, i.e. a 'stretch' or 'elongation', the modulus of elasticity involved is the one called Young's modulus.

The elastic behavior of a material, in a linear deformation, is shown by its 'elastic diagram', which is a plot of the elastic tension developed (as ordinate) vs. the amount of elongation (as abscissa). The classical work of Hooke on relatively homogeneous, non-living material (like steel or rubber) showed that in these substances the elastic diagram was a straight line. No tension is developed until the 'unstretched length' of the specimen is reached. Thereafter the tension is proportional to the elongation, i.e. the actual length minus the unstretched length,

$$T = A \times Y \times (\Delta L/L_0)$$

where T is the tension, A the cross-sectional area of the specimen, Y is Young's modulus, ΔL the elongation, and L_0 the unstretched length. This is Hooke's law.

Even with a homogeneous material, when the proportional elongation ($\Delta L/L$) exceeds the 'yield point' or 'elastic limit', the material begins to yield. Then the law no longer holds, and the tension does not increase as much with further elongation, so that the straight line of Hooke's law curves towards the axis of elongation. Such a curvature is seen in elastic diagrams

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even for homogeneous materials, unless a correction is applied for the fact that the cross-sectional area is not constant but diminishes as the specimen is stretched. Thus as the degree of stretch increases, homogeneous materials are almost always less able to resist further stretch, and their elastic diagrams characteristically bend towards the axis of elongation (the x-axis).

In marked contrast Roy (16), in 1880, observed that the distensibility curves of arteries did not follow Hooke's law but bent away from the axis of elongation more and more with increased pressure. Subsequent authors (Bramwell *et al.* (2), Hass (10), Lawton (13), Suter (19), Wilens (20)) have all found that the arterial wall resists stretch more strongly the more it is stretched.

This feature of the elastic diagram of arteries might be explained in one of two ways:

(*a*) While Hooke's law applies to homogeneous materials, the arterial wall is of a heterogeneous nature, containing two substances of widely different elastic moduli, namely, elastin with a Young's modulus of about 3×10^6 dynes/cm.^{2}, and collagen of about 1×10^9 dynes/cm.^{2} (Burton (3)).

(*b*) Alternatively, there might be some intrinsic elastic property of particular tissue elements in the wall, by which they resist elongation more as stretch increases. While it would be unusual to find such a property in a homogeneous material, though not impossible on the basis of a peculiar molecular organization, this might well be due to a special architectural arrangement of fibers in the tissue. (Lifting a weight hung from the middle of a stretched garden clothesline by pulling on the line is an example.) As we had undertaken a study of the distensibility of human iliac arteries, obtained at autopsy, with a view to deducing the elasticity of the tissues and the way in which this changed with age, it was desirable to settle, at the outset, this fundamental point as to which explanation applied.

The method of investigation that suggested itself was to obtain volume–pressure curves of the arteries, by the method already used in this laboratory by Nichol (14), and to transform the results into 'elastic diagrams' of tension vs. elongation by the use of the law of Laplace. (Tension = pressure $\times$ radius, for a cylindrical vessel.) It was known that digestion with formic acid (Hass (11)) would selectively remove the collagenous fibers, while leaving the elastic fibers relatively unchanged. If the elastic diagrams of vessels so treated could be obtained, comparison with the original curves might reveal the role of the collagenous fibers in the total elastic behavior. The alternative mode of investigation also appeared possible, i.e. to remove selectively the elastin fibers by digestion with crude trypsin, and again compare the new with the original elastic diagrams.

II. Apparatus and Methods

(*a*) *Measurement of Pressure–Volume Curves*

The external iliac artery was chosen as it was readily obtainable at autopsy and was a convenient size and because a length up to 3 or 4 cm. without branches was available. The distensibility was measured within a few hours

of death, or after storing of the vessels in the refrigerator in 1/10,000 saline solution of methiolate. No change in the distensibility curves was detectable during periods up to 10 days.

The apparatus (already described by Nichol (14)), consisted of a syringe connected by two three-way stopcocks to a metal adapter for the vessel, a reservoir of dye (useful for detecting the location of leaks), and a mercury manometer. A micrometer head connected to the piston of the syringe allowed small, but accurate, volume changes inside the vessel to be made. The resulting pressure was read on a direct, vertical mercury manometer. All joints were lubricated with Dow-Corning silicone stopcock grease and bubbles of air were carefully removed. An 'apparatus base line' was obtained by connecting the syringe to the manometer only. Then one end of the vessel was closed with a glass plug, and the other was attached to an open metal adapter which fitted tightly over the adapter of the apparatus, the vessel was connected to the syringe and manometer by turning the tap, and pressure–volume relations were measured. Both plug and adapter were made as large as possible to avoid the 'sausage-shape' which occurred at high pressures.

The vessel was collapsed by application of a 'negative' pressure until the apparatus base line was reached, indicating that the vessel was empty. Then the volume inside was slowly increased, and corresponding changes in pressure determined on the manometer. Volume–pressure graphs were drawn and then these were converted into the tension–length diagram by the law of Laplace (tension = pressure $\times$ radius). For this calculation, the radius was estimated from the volume in the vessel and the measured length of the segment. After curves from the fresh vessels were obtained, some of the vessels were treated with formic acid to remove collagen, while others were treated with crude trypsin to remove elastin, and the distensibility curves were again measured.

(*b*) *Formic Acid Digestion*

Hass (11) isolated elastic fibers by incubating aortae in 90% formic acid at 45° C. This digested the fibrous tissue much more rapidly than the elastic tissue. We found that the collagen was changed into a gelatinous material which initially clogged the vessel lumen, causing an increase in the cross-sectional area of the vessel wall. At a later stage (1 or 2 hours of digestion) this gelatinous residue disappeared; digestion was for periods ranging from 1 to 12 hours.

Chemical analysis was carried out by the Department of Pathological Chemistry at Victoria Hospital under the direction of Dr. R. Pearce. Weighed segments (200 mg.) of fresh and treated vessels were autoclaved four times in 1 ml. of water at 15 lb. pressure for 1 hour, the supernatant being poured off between each extraction. This removes only the collagen. Then the supernatant was analyzed for its hydroxyproline content, this being a good indication of the amount of collagen present (Block and Bolling (1)). Since hydroxyproline from elastin remains in the residue, use of the supernatant only eliminates this. The content of hydroxyproline in undigested tissue was

6.51 micromoles per g. of tissue. After 10 hours' digestion in formic acid it had fallen to 4.13 micromoles per g., a decrease of 36%. It is logical to assume that the 'cement-substance' bonds between the collagen fibers, anchoring them to other fibers, were broken down much earlier, thus rendering any fibers that were not yet digested unable to contribute to the elastic tension.

In an experiment on one vessel with which the time of digestion by formic acid was varied, the pressure–volume and the calculated tension–length diagrams showed no further change in the distensibility after 3 hours. In 19 vessels, the distensibility was measured when the vessels were fresh, and after digestion for 3 hours or more, or until the vessel began to leak. Histological sections of the vessels, before and after digestion with formic acid, indicated, by staining, a marked reduction in the collagen content of the vessel wall (Fig. 1).

(*c*) *Trypsin Digestion*

The crude trypsin we used (Nutritional Biochemicals Corporation) evidently contains an elastase (Hall (8)). Most authors (Harrow (9), Sizer (17)) state that trypsin and other proteolytic enzymes, except pepsin, have no effect on collagen. Pure trypsin does not affect elastin (Lansing (12), Franchi and de Robertis (6), Partridge *et al.* (15)) but some authors (Stein and Miller (18), Day (5), Gross (7)) have found, as we do, that crude trypsin causes loss of elasticity. Hass (10) found that the effect of crude trypsin digestion in an alkaline medium was to break up the fibers into globules held in position by their external sheath, rather than to make them thinner. Thus, functionally, only collagenous fibers remained, since the elasticity of the vessel wall depends on the continuity of the fibers in it.

We used 2 mg. of crude trypsin powder per ml. of standard phosphate–citrate buffer at pH 8.0. The vessels were incubated in this solution at 37° C. for periods ranging from 2 to 22 hours. Histological sections of a vessel before and after this treatment show that elastic tissue was removed (Fig. 1).

III. Results

(*a*) *Effects of Digestion on Pressure–Volume Curves*

Fig. 2 shows the experimental data on pressure vs. volume obtained on the iliac vessels of a 32-year-old male, plotted as the readings of the micrometer head of the injector (volume changes) vs. the manometer readings (pressure changes). The curves for the two 'fresh' vessels were practically identical. The vessel of one side was then digested for 1 hour in formic acid (collagen depleted), the other for 22 hours in trypsin (elastin depleted). The striking changes in the curves shown in Fig. 2 resulted. To collapse the fresh vessels so that the 'apparatus base line' was reached required only 5 to 10 mm. Hg 'negative' pressure in the vessel. The volume increase as the pressure was increased was steady, with a gradual decrease in distensibility as the wall was stretched more. After the elastin had been depleted by trypsin digestion, the collapse was remarkably sudden, only 1 or 2 mm. Hg negative pressure being required. This suggests that the elastic fibers of the intima are mainly

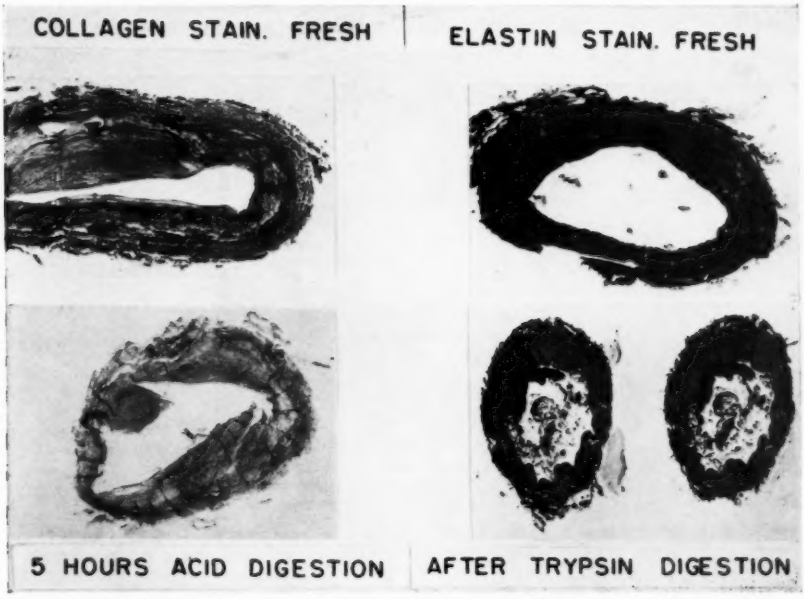

FIG. 1. Histological sections of iliac arteries, stained, on the left, for collagen (Van Gieson) and, on the right, for elastin (Weigert). The selective effects of the two types of digestion are obvious.

responsible for the resistance to deformation, and that the intima offers the main resistance to collapse of the vessel. This confirms the findings of Nichol (14). As the pressure was raised, the volume increased and very soon a steep slope, indicating low distensibility at high pressures, was evident. In contrast, the curve in the collagen-depleted vessel was nearly linear. The apparently increased resistance to collapse over that of the fresh vessel is undoubtedly due to the gelatinous dissolved collagen partially choking the lumen.

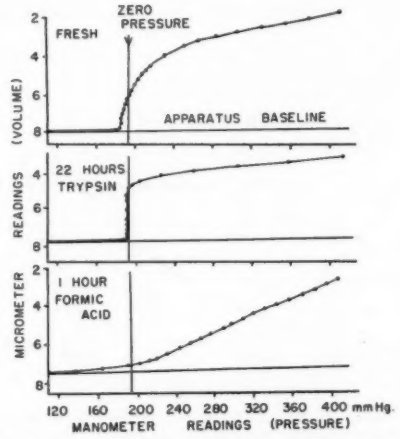

FIG. 2. Changes in the shape of volume–pressure curves of iliac arteries produced by differential digestion of elastin (by trypsin) and of collagen (by formic acid).

(b) Tension–Length Diagrams

Fig. 3 shows the P–V diagrams of Fig. 2 transformed into the T–L diagrams by the law of Laplace ($T = P \times r$). The curve from the fresh vessel had the characteristic shape described by Roy and others, while that from the 'elastic' vessel was practically linear until it had been stretched to more than twice its initial diameter (three to four times in some cases). In contrast, that from the 'collagenous' vessel immediately became steep. In Fig. 4, to compare vessels of different unstretched diameter, all length measurements have been expressed as a percentage of the initial circumference. This does not alter the shape of the curves, but merely changes their position relative to one another.

In the T–L diagrams of Fig. 4, the initial slope (i.e. at low pressure) of the distensibility curve of the fresh vessel is approximately equal to the slope of the distensibility curve of the 'collagen-digested' artery, while the final slope (at high pressure) of the curve from the fresh vessel agreed very well with the slope of the distensibility curve of the 'elastin-digested' vessel. The curves were not superimposed at these points, partly because of a difference in lumen diameter (see below) and partly because of an incomplete removal of the tissue element digested in each case. After depletion of collagen by formic acid, the 'unstretched' diameter of the vessels was considerably less than in the

untreated vessels, while after elastin depletion, the diameter increased. This seems to indicate that even when the vessel is unstretched by the blood pressure, the elastic fibers are under some tension, and are pulling in the collagen fibrous 'jacket'. This view would explain the classical results on the changes in volume–pressure relations in the aorta with age and with degeneration of the elastic fibers, which indicate a considerable increase in the unstretched diameter of the aorta with age.

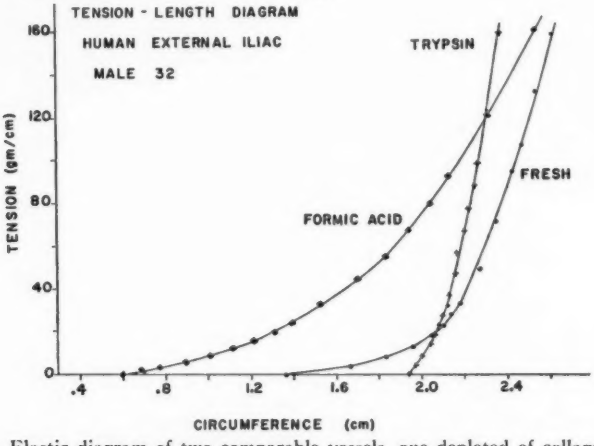

FIG. 3. Elastic diagram of two comparable vessels, one depleted of collagen (formic acid digestion) the other of elastin (trypsin).

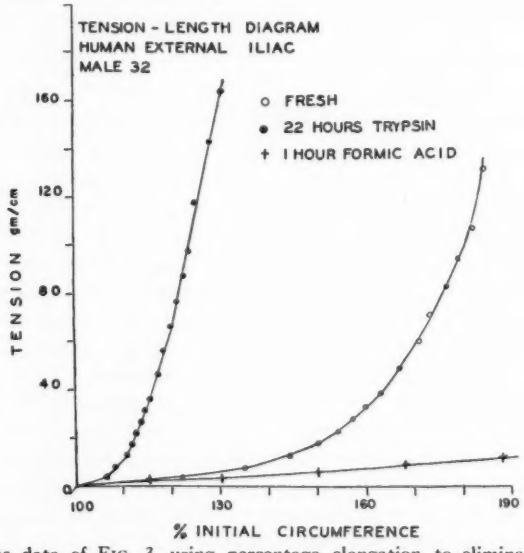

FIG. 4. The data of FIG. 3, using percentage elongation to eliminate changes in unstretched radius of the vessels.

Fig. 5 shows the progressive change of the elastic diagram with the duration of digestion of collagen. The effect is obviously on the 'high-pressure slope' of the curve, rather than on the initial slope at zero pressure. There was no further significant change with digestion of more than 3 hours.

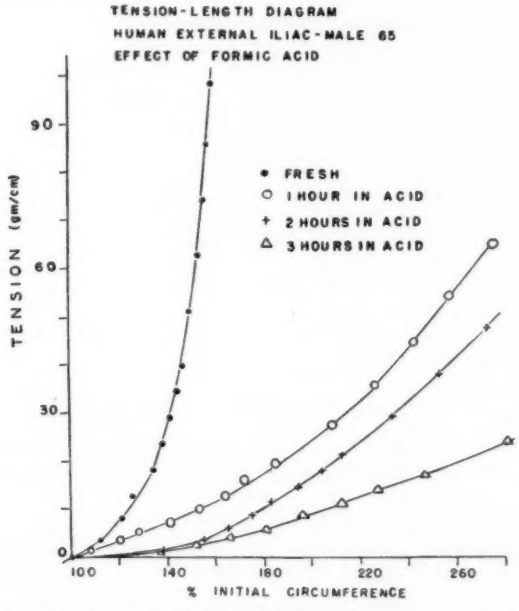

FIG. 5. Progressive changes in the elastic diagram of an iliac artery with the duration of digestion with formic acid (collagen depletion).

Fig. 6 summarizes the results on all of the vessels by using mean values. The broken lines indicate the standard error of estimate. The final slope of the curves after elastin depletion (trypsin digestion) agrees well with the slope of the curve for fresh vessels at high pressure, while the slope of the almost linear curve after collagen depletion (formic acid digestion) is lower than the initial slope of the fresh vessels. This may be either because the formic acid digestion, though not digesting the elastin fibers, has weakened their anchorage to collagen fibers, or because even at very low pressures some collagen fibers are contributing to the total elastic tension.

(*c*) *Activity of Smooth Muscle—Experiments with Adrenaline*

Two experiments done on vessels procured and measured within 2 hours of death showed that there was no contraction of smooth muscle after injection of a concentrated solution of adrenaline; at constant volume there was no increase in pressure inside the vessel when the adrenaline was injected. This indicates that the smooth muscle that is present, during life, in the external iliac, plays no part in the distensibility of the isolated vessels studied in this

research. Moreover, there is good evidence (Burton (3)) that the contribution of smooth muscle to the elastic properties of living blood vessels is very small. The function of the smooth muscle is to produce 'active tension', relatively independent of stretch, rather than 'elastic tension' which is proportional to stretch.

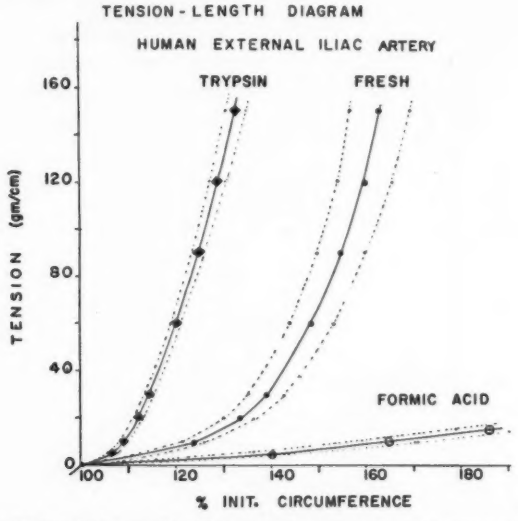

FIG. 6. The elastic diagram of an iliac artery representing the mean of the results on nine vessels of the young to middle age group (20–50 years). The broken lines indicate the standard error.

IV. Discussion

Calculation of Results in Terms of 'Elastance' of the Wall

It is not possible to obtain accurate values for the Young's modulus of the elements of the wall because we cannot estimate well the cross-sectional area of the fibers. However, we can define an 'elastance' as the product of the Young's modulus and the cross-sectional area ($Y \times A$). This is equal to the rate of change of the elastic tension with respect to the relative elongation, $(1/L_0 \times dt/dL)$, and therefore is given by the slope of the curves of the elastic diagrams. The 'elastance' tells us the force that would be developed if the length of the fibers were doubled. Table I shows the mean values of elastance for all the iliac arteries, and the changes in elastance produced by depletion of the collagen, or of the elastin, by selective digestion. The values are calculated from the slope of the elastic diagrams for three different conditions of distension of the vessel. The 'initial slope' gives the elastance when the vessel is stretched only slightly from the unstretched condition, and this would apply at very low pressures of blood in the vessel. The elastance was also calculated at points on the elastic diagrams corresponding to a pressure of 100 mm. Hg for the physiological range of arterial pressure. The final slope, where the curves had become linear, represents the elastance when all of the

fibers have now reached their unstretched length and are all contributing to the total elastic tension, and might apply in hypertension. The vessels used for Table I were from patients in the 40–60 age group. Periods of digestion varied, for formic acid, from 1 to 12 hours, and for crude trypsin, from 2 to 22 hours.

TABLE I

EFFECT OF FORMIC ACID AND OF TRYPSIN ON ELASTANCE OF WALL

Elastances are given in 10^5 dynes per cm. length of vessel for 100% increase in diameter

Condition of wall	No. of specimens	Mean values of elastance ± standard error of mean		
		From initial slope	From slope at 100 mm. Hg	From final slope
Untreated	9	0.229 ± 0.025	4.69 ± 0.50	9.35 ± 0.95
After trypsin, i.e. elastin-depleted	9	0.718 ± 0.234	4.60 ± 0.50	8.30 ± 0.90
After formic acid, i.e. collagen-depleted	9	0.170 ± 0.085	0.71 ± 0.42	1.30 ± 0.45

STATISTICAL ANALYSIS OF DIFFERENCES

P-values by *t*-test. Figures in parentheses indicate that there was no significant difference

	From initial slope, i.e. for low pressures	From slope at 100 mm. Hg, i.e. physiological range	From final slope, i.e. for high pressures
Intact vs. collagen-depleted	(0.5)	<0.01	<0.01
Intact vs. elastin-depleted	0.05	(0.9)	(0.5)
Collagen-depleted vs. elastin-depleted	<0.05	<(0.01)	<0.01

The mean values and the statistical analysis given under Table I show that:

(*a*) The depletion of collagen in the vessel wall does not significantly alter the initial slope, i.e. the elastance at low pressure.

(*b*) In contrast, depletion of collagen very markedly and significantly reduces the elastance at high pressures. Therefore the elastance at high pressures is mainly due to collagenous fibers. The elastance at 100 mm. Hg pressure is also markedly reduced, and therefore the tension at physiological pressures in the iliac artery is largely, but not exclusively, due to collagenous fibers.

(*c*) On the other hand, depletion of elastin does not significantly alter the elastance at high pressures, or at 100 mm. Hg, while it significantly alters the elastance at very low pressures. The elastic fibers are therefore chiefly responsible for the tension at low pressures, but play a minor part at higher pressures.

The results show clearly that the non-linear shape of the elastic diagram of these arteries, and the property of resisting distension more strongly the

greater the pressure, are due to the heterogeneous nature of the wall. At low pressures, only the elastic fibers have reached their unstretched length, but as the pressure is increased, more and more of the collagenous fibers of the 'fibrous jacket' reach their unstretched length. At physiological arterial pressures and higher pressures, elastic tension is predominantly due to these collagenous fibers.

Though in the physiological range of pressure, the elastin fibers contribute only a small part of the total tension, their role is not unimportant. It has been shown (Burton (3)) that without the elastic fibers, stability of the vessel wall, with a fine gradation of vasoconstriction due to the contraction of smooth muscle, would not be possible.

For the purposes of further research on the changes of elasticity of the vessels with age, these results are quite conclusive. The initial slope of the elastic diagrams can be utilized as a reliable index of the state of the elastin fibers, such as the fibers of the elastica intima, and the final slope can be used as an index of the state of the collagenous fibers. It will be our aim to correlate the elastances so obtained with the histopathological findings in the process of aging.

V. Acknowledgments

We are greatly indebted to Professor J. Fisher at Victoria Hospital, Dr. Looney at St. Joseph's Hospital, and Dr. J. Paterson at Westminster Hospital for their help in obtaining autopsy specimens. Dr. R. Pearce, of the Department of Pathological Chemistry, supervised the chemical measurements, and Dr. R. Buck contributed the histological examinations. We wish to thank Dr. J. Nichol for advice, and Mrs. D. Elston for much technical assistance.

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Notes to Contributors

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(i) ***General***

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Style, arrangement, spelling, and abbreviations should conform to the usage of this journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed.

Manuscripts and illustrations should be carefully checked before they are submitted. Authors will be charged for unnecessary deviations from the usual format and for changes made in the proof that are considered excessive or unnecessary.

(ii) ***Abstract***

An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

(iii) ***References***

References should be listed **alphabetically by authors' names,** numbered, and typed after the text. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should not be given and initial page numbers only are required. The names of periodicals should be abbreviated in the form given in the most recent *List of Periodicals Abstracted by Chemical Abstracts.* All citations should be checked with the original articles, and each one referred to in the text by the key number.

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Tables should be numbered in roman numerals and each table referred to in the text. Titles should always be given but should be brief; column headings should be brief and descriptive matter in the tables confined to a minimum. Vertical rules should be used only when they are essential. Numerous small tables should be avoided.

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